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(54) Title: THERMOSTABLE DNA TOPOISOMERASE V

(57) Abstract

A type 1 topoisomerase, designated topoisomerase V, has been isolated and substantially purified from the halophilic thermophilic methanogen bacterium *Methanopyrus kandleri*. The topoisomerase was purified by a process including the steps of lysing cells of *M. kandleri* to form a lysate, treating the lysate with polyethyleneimine to form a precipitate and a supernatant, precipitating the polyethyleneimine supernatant with ammonium sulfate, chromatographing the ammonium sulfate precipitate on phosphocellulose to produce a phosphocellulose eluate, chromatographing the phosphocellulose eluate on heparin to produce a heparin eluate, and chromatographing the heparin eluate on a column capable of separating proteins by molecular size therein to produce a substantially purified thermostable DNA topoisomerase V. Topoisomerase V can relax DNA and can unlink DNA by reducing the linking number of closed circular DNA. Topoisomerase V is active over a wide range of temperatures and ionic conditions and does not require magnesium or ATP for its activity. It is recognized by anti-human topoisomerase I antibody.

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THERMOSTABLE DNA TOPOISOMERASE V

BACKGROUND OF THE INVENTION

This invention is directed to a thermostable type I DNA topoisomerase V from the thermophilic prokaryote Methanopyrus kandleri, methods for its purification, and methods for its use.

The intertwining of the two strands of the DNA helix presents a number of topological problems which the cell must overcome in order to regenerate, recombine, and express its genetic information (M. Gellert, Annu. Rev. Biochem. 50:879-910 (1981); J. C. Wang, Annu. Rev. Biochem. 54: 665-697 (1985); A. Maxwell and M. Gellert, Adv. Protein Chem. 38: 68-107 (1986); N. Osheroff, Pharmac. Ther. 41: 223-241 (1989); J.C. Wang et al., Cell 62: 403-406 (1990); J. C. Wang, J. Biol. Chem. 266: 6659-6662 (1991); A. Kornberg and T. A. Baker, in DNA Replication (2d ed.), W.H. Freeman and Company, New York, 1992, pp. 379-401; J. C. Wang and L. F. Liu, in DNA Topology and Its Biological Effects (N.R. Cozzarelli and J.C. Wang, eds., Cold Spring Harbor Laboratory Press, New York, 1990, pp. 321-340). Consequently, the enzymes modulating the topological state of nucleic acids, known as DNA topoisomerases, play a crucial role in controlling the physiological functions of DNA.

To simplify the later description, a few definitions relevant to DNA topology are useful. Supercoiling is typical in closed circular DNA (ccDNA) because of the topological linkage of the two complementary strands. The linking number, Lk, is the quantitative measure of this linkage: it is the algebraic number of times one strand crosses the surface stretched

over the other strand. The Lk value is a topological invariant for ccDNA; there is no way it can be changed without introducing chain scissions. The number of supercoils can be defined as

$$\Delta Lk = Lk - N/\gamma_0$$

where N is the number of base pairs in a DNA and γ_0 is the number of base pairs per turn of the double helix under given ambient conditions. Specific linking difference, or superhelical density, is defined as

$$\sigma = \gamma_0 \Delta Lk/N$$

If $\Delta Lk > 0$, DNA is called positively supercoiled; if $\Delta Lk < 0$, DNA is negatively supercoiled. A consequence of negative supercoiling is that the DNA helix is more easily unwound, i.e., the strands are more readily separated, whereas positive supercoiling, by tightening the pitch of the helix, would make unwinding more difficult.

Any changes in the Lk value are resolved into its two geometrical components (J.H. White, Am. J. Math. 91: 693-728 (1969))

$$\Delta Lk = \Delta Tw + Wr$$

where ΔTw is the difference in the axial twist of either strand about the axis of the double helix, Wr is the quantity related to supercoiling and is determined by the spatial shape of the axis of the double helix (for detailed discussions on general aspect of DNA topology, see DNA Topology and Its Biological Effects (N.R. Cozzarelli and J.C. Wang, eds., Cold Spring Harbor Laboratory Press, New York, 1990)).

DNA transformations performed by DNA topoisomerases are accomplished by the cleavage of either a single strand or both strands. The unit change in the Lk upon such transformations is the best operational distinction between the two classes of topoisomerases (P.O. Brown & N.R. Cozzarelli, Science 206: 1081-1083 (1979)). DNA topoisomerases whose reactions proceed via

a transient single-stranded break and changing the Lk in steps of one are classified as type 1, while enzymes whose reactions proceed via double-stranded breaks and changing the Lk in steps of two are classified as type 2.

Members of type 2 topoisomerase family include DNA gyrase, bacterial DNA topoisomerase IV, T-even phage DNA topoisomerases, eukaryotic DNA topoisomerase II, and thermophilic topoisomerase II from Sulfolobus acidocaldarius (see reviews cited above; A. Kikuchi et. al., Syst. Appl. Microbiol. 7: 72-78 (1986); J. Kato et. al., J. Biol. Chem. 267: 25676-25684 (1992); W.M. Huang in DNA Topology and Its Biological Effects (N.R. Cozzarelli and J.C. Wang, eds., Cold Spring Harbor Laboratory Press, New York, 1990), pp.265-284; T.-S Hsieh in DNA Topology and Its Biological Effects (N.R. Cozzarelli and J.C. Wang, eds., Cold Spring Harbor Laboratory Press, New York, 1990), pp.243-263)). The coding sequences of a dozen or so type 2 enzymes have been determined, and the data suggest that all these enzymes are evolutionarily and structurally related. Topological reactions catalyzed by type 2 topoisomerases include introduction of negative supercoils into DNA (DNA gyrase), relaxation of supercoiled DNA, catenation (or decatenation) of duplex circles, knotting and unknotting of DNA.

The family of type 1 topoisomerases comprises bacterial topoisomerase I, E. coli topoisomerase III, S. cerevisiae topoisomerase III (R.A. Kim & J.C. Wang, J. Biol. Chem. 267: 17178-17185 (1992)), the type 1 topoisomerase from chloroplasts that closely resembles bacterial enzymes (J. Siedlecki et. al., Nucleic Acids Res. 11: 1523-1536 (1983)), thermophilic reverse gyrases (A. Kikuchi in DNA Topology and Its Biological Effects (N.R. Cozzarelli and J.C. Wang, eds., Cold Spring Harbor Laboratory Press, New York, 1990), pp.285-298); C. Bouthier de la Tour et. al. J. Bact. 173: 3921-3923

(1991)), thermophilic D. amylolyticus topoisomerase III (A.I. Slesarev et. al., J. Biol. Chem. 266: 12321-12328 (1991), nuclear topoisomerases I and closely related enzymes from mitochondria and poxviruses (N. Osheroff, Pharmac. Ther. 41: 223-241 (1989)). With respect to the mechanism of catalysis these topoisomerases can be divided into two groups. Group A consists of enzymes that are single-strand-specific and form a transient covalent complex with the 5'-phosphoryl termini (prokaryotic type 1 topoisomerases and S. cerevisiae topoisomerase III). Group B includes type 1 topoisomerases that operate on duplex DNAs and bind covalently to the 3'-phosphoryl termini (nuclear topoisomerases I, enzymes from mitochondria and poxviruses commonly called eukaryotic topoisomerases I). Type 1 topoisomerases can carry out the following topological reactions; they relax supercoiled DNA (except of reverse gyrases), catenate (or decatenate) single-stranded circular DNAs or duplexes providing that at least one of the molecules contains a nick or gap, interact with single-stranded circles to introduce topological knots (type 1-group A topoisomerases). Reverse gyrase, belonging to type 1-group A topoisomerases, is the only topoisomerase shown to be able to introduce positive supercoils into ccDNA.

Research on DNA topoisomerases has progressed from DNA enzymology into developmental therapeutics. Bacterial DNA topoisomerase II is an important therapeutic target of quinolone antibiotics; mammalian DNA topoisomerase II is the cellular target of many potent antitumor drugs (K. Drlica, Microbiol. Rev. 48: 273-289 (1984) and Biochemistry 27: 2253-2259 (1988); B.S. Glisson & W.E. Ross, Pharmacol. Ther. 32: 89-106 (1987); A.L. Bodley & L.F. Liu, Biotechnology 6: 1315-1319 (1988); L.F. Liu, Annu. Rev. Biochem. 58: 351-375 (1989)). These drugs, referred to as topoisomerase II poisons, interfere with the breakage-rejoining reaction of type II

topoisomerases by trapping a key covalent reaction intermediate, termed the cleavable complex. Mammalian type 1-group B topoisomerase has been shown to be the target of camptothecin (CPT), a plant alkaloid with strong antitumor activity. CPT and its derivatives also trap a putative covalent reaction intermediate, the cleavable complex. This type of reversible DNA damage is lethal to proliferating cells and is responsible for the antitumor activity of topoisomerase poisons (Y.-H. Hsiang et al., J. Biol. Chem. 260: 14873-14878 (1985); Y. Hsiang et. al., Cancer Res. 49: 5077-5082 (1989); C. Holm et. al., Cancer Res. 49: 6365-6368 (1989), P. D'Arpa et. al. Cancer Res. 50: 6919-6924 (1990); A.Y. Chen et. al. Cancer Res. 51: 6039-6044 (1991)). Increased attention to CPT and its derivatives as the most promising anticancer agents currently in clinical trials (W.J. Slichenmyer et. al., J. Natl. Cancer Inst. 85: 271-287 (1993); U.S. Pat. No. 5,106,742 (camptothecin analogues as potent inhibitors of topoisomerase I);U.S. Pat. No. 5,122,526 (camptothecin and analogues thereof and pharmaceutical compositions and method using them)) resulted in isolation several CPT-resistant cell lines and a CPT-resistant mutant of human topoisomerase I has been characterized (H. Tamura et. al., Nucleic Acids Res. 19: 69-75 (1991)). Through the use of a yeast strain in which yeast type 1-group B topoisomerase is replaced by its human counterpart, other CPT-resistant mutants of the human enzyme have been isolated (P. Benedetti et. al., in Drug Resistance as a Biochemical Target in Cancer Chemotherapy (T. Tsuruo, M. Ogawa, & S.K. Carter, eds., Academic Press, San Diego, CA, 129, 1991)). Further identification of the mutation sites of CPT-resistant type 1-group B topoisomerases is potentially useful for modeling novel derivatives acting against CPT-resistant tumor cells.

To date type 1-group B topoisomerases have been found only in eukaryotes. One might expect that the

finding of a prokaryotic counterparts to eukaryotic type 1-group B topoisomerases would be viewed with great interest by pharmacologists and clinicians as well. Whereas it would be important to exploit the common features of type 1-group B topoisomerases, it would be equally important to exploit the differences among them for modeling novel drugs. Also the new organisms harboring type 1-group B topoisomerases would be of clinical interest as potential sources of the natural inhibitors of those enzymes. For example, in E. coli, the miniF plasmid CcdB protein, like quinolone antibiotics and antitumoral drugs, promotes DNA gyrase-mediated double-stranded DNA breakage (P. Bernard & M. Couturier, J. Mol. Biol. 226: 735-745 (1992)).

Another aspect of medical utility of type 1-group B topoisomerases is the identification of the human Scl-70 antigen as DNA topoisomerase I. Scleroderma (progressive systemic sclerosis) patients may produce high titer autoimmune antibody directed against human topoisomerase I (J.H. Shero et. al., Science 231: 737-740 (1986)). The availability of cloned human topoisomerase I enables the development of methods for rapid screening for the presence of these autoantibody in patient sera (P. D'Arpa et al. Proc. Natl. Acad. Sci. USA 85: 2543-2547 (1988); U.S. Pat. No. 5,070,192 (cloned human topoisomerase I: cDNA expression and use for autoantibody detection)). Remarkably, type 1-group B topoisomerases of higher plants are recognized by human anti-topoisomerase autoantibody, despite the divergence of the kingdoms (P.F. Agris et. al., Exp. Cell Res.189: 276-279 (1990)). It is conceivable that human autoantibody could recognize type 1-group B topoisomerases from prokaryotic organisms as well. Using human antibody on prokaryotic systems immunoprecipitations, competitive binding assays, cellular function studies, and probing expression libraries could be accomplished. The use of human anti-topoisomerase

antibody as probes of prokaryotic type 1-group B topoisomerase structure may be important in further understanding the interaction of human topoisomerase I and cancer chemotherapeutic agents. Finally, such cross-reactivity would have clinical significance for autoimmunity as well. Sera of scleroderma patients targeted at least 6 independent epitopes on human topoisomerase I. Molecules of cDNA comprising a part of the cDNA sequence of human topoisomerase I which encode at least one epitope for autoantibody to human topoisomerase I are available (P. D'Arpa et al., Arthritis Rheum. 33: 1501-1511 (1990)). Other type 1-group B topoisomerases reacting with anti-human topoisomerase I antibody could be used in the same way. It would be important in tracking the emergence of autoimmune antibody against particular epitopes during the progression of disease.

One of the driving forces behind molecular biology is the successful utilization of enzymes as reagents. Mesophilic type 1-group B topoisomerases are widely used for analysis of DNA supercoiling, DNA conformation, transcription in vitro, and chromatin reconstitution (M.D. Frank-Kamenetskii, in DNA Topology and Its Biological Effects (N.R. Cozzarelli and J.C. Wang, eds., Cold Spring Harbor Laboratory Press, New York, 1990), pp.185-215; Y. Zivanovic et. al., J. Mol. Biol. 214: 479-495)). The availability of thermophilic type 1-group B topoisomerases simplifies significantly the manipulation of DNA conformation. Reverse gyrase is becoming an indispensable tool for preparation of positively supercoiled DNA, while D. amylolyticus topoisomerase III is a convenient tool for preparation of ccDNA with different degree of negative supercoiling (A.I. Slesarev et. al. J. Biol. Chem. 266: 12321-12328 (1991)).

Chromatin reconstitution in vitro is usually done at 0.5-1.0 M while eukaryotic topoisomerase I is inhibited

by 0.2 M and higher concentration of NaCl (P.A. Der Garabedian et al., Biochemistry 30: 9940-9947 (1990)). At 0.2 M NaCl nucleosome assembly is very inefficient, so there is a need in topoisomerase working in a wide range of ionic strength. Moreover, recent progress in this field increases the need for thermostable topoisomerases with properties akin to those of eukaryotic topoisomerase I (J. Bashkin et. al. Biochemistry, 32: 1895-1898 (1993)).

Other applications of topoisomerases may exploit their ability to cleave DNA at specific sites ("specific endonucleases"), their ability form a covalent complex with DNA, their substrate selectivity. Of potential interest is a topoisomerase religation half-reaction. Type 1-group B topoisomerases catalyze the in vitro ligation of nonhomologous DNA fragments lacking any sequence homology or complementarity (M.D. Been & J.J. Champoux, Proc. Natl. Acad. Sci. USA 78: 2883-2887 (1981)). Pan et al. disclosed a method for ligation of artificial substrates that bear a tyrosine residue on the 3'-PO_4 of an appropriate oligonucleotide with mammalian topoisomerase I (J. Biol. Chem. 268: 3683-3689 (1993)).

Preparation and analysis of specific nucleotide and protein sequences constitutes a basis of current molecular biology, biotechnology, and molecular medicine. Enzymatic DNA sequencing (F. Sanger, Proc. Natl. Acad. Sci. USA 74: 5463-5467 (1977)) using Sequenase enzyme (US Biochemical, Cleveland, OH) is currently the most popular method to obtain genetic information. Any improvement in the procedure that will eliminate labor-consuming steps or increase sensitivity, is of great demand in the field. It is generally accepted that, despite problems with DNA denaturation and reassociation, the use of double-stranded DNA for sequencing is preferable to the construction and use of single-stranded DNA. The denaturation of conventionally purified ccDNA involves alkali treatment

that is hazardous for DNA followed by time and labor-consuming DNA precipitation, during which reassociation can occur (S.M. Adams et. al. Focus 13: 56-58 (1991); D.F. Barker, Biotechniques 14: 168-169 (1993)). This results in fewer readable bases. Sequencing at higher temperatures can eliminate reassociation, but results in a higher error rate.

Thus, there is a need for a reliable technology of plasmid template preparation for sequencing compatible with popular sequencing protocols.

Polymerase chain reaction (R.K. Saiki et. al., Science 230: 1350-1354 (1985); H.A. Erlich, ed., PCR technology: Principles and applications for DNA amplification (Stockton Press, New York, 1989)) is the basic technique that allows amplification of pieces of DNA starting from tiny amounts of material with limited knowledge about its exact primary structure. Its development and refinement continue at a rapid pace. Another chain reaction introduced in 1990 (European Patent Application EP 88311741.8; F. Barany, Proc. Natl. Acad. Sci. USA 88: 189-193 (1990)) is based on ligase and is becoming a powerful tool in medical diagnostics. The success of these techniques was due to the use of thermostable enzymes. However, when PCR or LCR amplification is performed on a plasmid template, poor denaturation of ccDNA may result in a failure to detect products (E.C. Lau et al., Biotechniques 14: 378 (1993)). Therefore, these techniques also need a procedure of plasmid template preparation for reliable primer annealing.

One of the solutions of the above problem consists in the substitution of DNA denaturation with a special and simple topological treatment with a thermostable enzyme(s) that will allow a primer to anneal

to the double-stranded DNA with same efficiency as to the single-stranded DNA. The effect is based on the topological destabilization (unlinking) of the double helix that can be varied in a wide range. The limit products of the unlinking are ccDNA with a few links or even single-stranded complementary rings. Such DNA, called form V, can contain 10 to 40% single-stranded regions at room temperature and will melt easily at elevated temperature (U.H. Stettler et. al., J. Mol. Biol. 131: 21-40 (1979)). The non-template strand of the duplex will not hinder subsequent elongation and moreover it will create a topological force in favor of elongation. The method is equally applicable to any procedure that involve primer annealing and/or elongation, i.e., enzymatic ccDNA sequencing, PCR, LCR, hybridization probe preparation, etc.

There are several enzymatic procedures for preparation of highly underwound ccDNA molecules (M. Iwabuchi et al., J. Biol. Chem. 258: 12394-12404 (1983); A.M. Wu et al., Proc. Natl. Acad. Sci. USA 80: 1256-1260 (1983); T.A. Baker et al., Cell 45: 53-64 (1986); B.F. Pugh & M.M. Cox, J. Biol. Chem. 262: 1326-1336 and 1337-1343 (1987); C.A. Parada & K.J. Marians, J. Biol. Chem. 264: 15120-15129 (1989); B.F. Pugh et al., J. Mol. Biol. 205: 487-492 (1989)). However, the procedures require multienzyme complexes of at least two different enzymes and specific buffer conditions. In addition, the enzymes used in the above procedures are thermolabile and can not be used in PCR or LCR.

Slesarev et al. disclosed that thermostable type 1-group A topoisomerase III from *Desulfurococcus amylolyticus* can alone substantially reduce the Lk value of ccDNA and generate highly unwound forms of ccDNA in the linear DNA melting range (J. Biol. Chem. 266: 12321-12328 (1991)). However, this topoisomerase is Mg^{2+}-dependent,

ineffective at low salt conditions, and inhibited by single-stranded DNA. In addition, it needs very high temperature to be active on relaxed and positively supercoiled DNA. These properties of Dam topoisomerase III will interfere with the standard buffer conditions used in enzymatic DNA sequencing, PCR or LCR and enzyme will be inhibited by the reaction products.

Thus, there is a need for a thermostable (ATP, Mg^{2+})-independent relaxing DNA topoisomerase that is not inhibited by single-stranded DNA, equally active on positively and negatively supercoiled DNA, active through a wide range of temperatures and a wide range of salt conditions, to allow the performance of these manipulations on DNA more conveniently and more efficiently.

SUMMARY

A thermostable type 1-group B DNA topoisomerase has been isolated and purified to substantial homogeneity from the hyperthermophilic methanogen Methanopyrus kandleri. This topoisomerase, designated topoisomerase V, is the first type 1-group B topoisomerase known from a prokaryote. The substantially purified topoisomerase is substantially free of other enzymes acting on DNA. The purified topoisomerase is a single chain polypeptide with an estimated molecular mass of about 110,000 daltons, based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Another aspect of the invention is a process for purifying thermostable topoisomerase V. The process comprises:

(1) lysing cells of M. kandleri to form a lysate;

(2) treating the lysate with polyethyleneimine to form a precipitate and a supernatant;

(3) precipitating the polyethyleneimine supernatant with ammonium sulfate;

(4) chromatographing the ammonium sulfate precipitate on phosphocellulose to produce a phosphocellulose eluate;

(5) chromatographing the phosphocellulose eluate on heparin to produce a heparin eluate; and

(6) chromatographing the heparin eluate on a column capable of separating proteins by molecular size therein to produce a substantially purified thermostable DNA topoisomerase V.

Another aspect of the invention is a method of relaxation of supercoiled DNA comprising treating a supercoiled DNA selected from the group consisting of positively supercoiled DNA and negatively supercoiled DNA with the topoisomerase V of the present invention at a temperature below the melting temperature of the linear form of treated closed circular DNA and ionic conditions that allow the enzyme to bind to DNA and catalyze the relaxation reaction to produce at least partially relaxed DNA.

Yet another aspect of the invention is a method of unlinking closed circular DNA comprising treating closed circular DNA with the topoisomerase V of the present invention at a temperature at least as high as the melting range of the linear form of treated DNA and ionic conditions that allow the enzyme to bind to DNA and catalyze the unlinking reaction to produce DNA with a linking number lower than the linking number of the DNA before treatment.

Another aspect of the invention is a complex comprising the topoisomerase of the present invention non-covalently bound to DNA.

Still another aspect of the invention is a complex comprising the topoisomerase of the present invention covalently linked to the 3'-end of a DNA strand. The topoisomerase can be covalently linked to the 3'-end of a broken strand of open circular DNA.

Another aspect of the invention is a method for covalent complex formation comprising:

(1) incubating the topoisomerase of the present invention with DNA under ionic conditions allowing the binding of the topoisomerase to the DNA and the breaking of one strand by the topoisomerase; and

(2) denaturing of the topoisomerase leaving a covalent complex between the topoisomerase and the DNA.

Yet another aspect of the invention is a method for use of topoisomerase V of the present invention as a specific endonuclease comprising:

(1) incubating the topoisomerase of the present invention with DNA under ionic conditions allowing the binding of the topoisomerase to the DNA and the breaking of one strand by the topoisomerase at a specific sequence; and

(2) then denaturing the topoisomerase yielding cleaved DNA.

Another aspect of the invention is a method for producing an activated DNA substrate with at least a single amino acid residue covalently bound to the 3'-PO_4 terminus of a DNA strand adjacent to the recognition site of the topoisomerase of the present invention, comprising:

(1) incubating the topoisomerase with DNA under ionic conditions allowing the binding of the topoisomerase

to DNA and the breaking of one strand by the topoisomerase at a specific sequence; and

(2) then denaturing the topoisomerase and hydrolyzing the topoisomerase with a nonspecific protease.

Partial amino acid sequences are known for topoisomerase V, including the amino-terminal amino acid sequence and sequences of fragments produced by the proteolytic enzyme endoproteinase Lys-C. Accordingly, also within the scope of the invention are thermophilic type 1-group B DNA topoisomerases possessing an amino-terminal amino acid sequence selected from the group consisting of:

(1) the amino-terminal amino acid sequence of M. kandleri topoisomerase V; and

(2) an amino-terminal amino acid sequence related to the amino-terminal amino acid sequence of M. kandleri topoisomerase V by one or more conservative amino acid substitutions.

Similarly, within the scope of the invention are thermostable type 1-group B DNA topoisomerases possessing at least one amino acid sequence selected from the group consisting of:

(1) the sequence K-S-D-T-E-T-I-E-T

(2) the sequence K-P-E-L-P-Y-V-A-V-P-P-H-M-A-E-R-A-R-R-V-L-T-R-E-D-D-L-A-X-D-V-X-A

(3) the sequence K-R-V-P-R-A-X-X-G-X-X-F-D-R-L

(4) the sequence K-S-G-R-Q-E-R-S-E-E-E-E-K-E-E-L-E-R-K-V-G-E-G-R-A-R-R-L-I-E-Y-F-G-S-A;

(5) the sequence K-Y-G-S-A-S-X-X-R-R-L-P-X-E-E-X-R-E-L-G-F-X-D-D-R; and

(6) a sequence related to the sequences recited in (1)-(5) by one or more conservative amino acid substitutions.

Another aspect of the present invention is antibody specifically binding the substantially purified thermostable DNA topoisomerase of the present invention. The antibody can be a monoclonal antibody.

Another aspect of the invention is an immunoreactive complex of topoisomerase V of the present invention with anti-human topoisomerase I antibody.

Another aspect of the invention is an immunoassay for the detection or determination of the topoisomerase of the present invention with anti-human topoisomerase I antibody comprising the steps of:

(1) reacting the topoisomerase with anti-human topoisomerase I antibody; and

(2) detecting and/or determining the topoisomerase V by detecting and/or determining an antigen-antibody complex between the topoisomerase and the antibody.

Other antibodies are also aspects of the present invention. They include: (1) antibody specifically binding a fragment of about 75,000 daltons of the substantially purified topoisomerase of the present invention originating from the amino-terminus of the topoisomerase protein; and (2) antibody specifically binding a fragment of about 33,000 daltons of the substantially purified topoisomerase of the present invention originating from the carboxyl-terminus of the topoisomerase protein.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects and advantages of the present invention will become better understood with reference to the following description, appended claims, and the accompanying drawings where:

Figure 1 is a graph of A_{280} and NaCl concentration for the eluate from the phosphocellulose column in the purification of Methanopyrus kandleri topoisomerase V, together with a photograph of a silver-stained 4-15% gradient polyacrylamide gel showing the protein compositions of fractions in the eluate and indicating the fractions taken as Fractions IVa and IVb;

Figure 2 is a graph of A_{280} and NaCl concentration for the eluate from the heparin chromatography of Fraction IVa, together with a photograph of a Coomassie blue-stained 4-15% gradient polyacrylamide gel showing the protein compositions of fractions in the eluate and indicating the fractions taken as Fraction Va;

Figure 3 is a graph of A_{280} and NaCl concentration for the eluate from the heparin chromatography of Fraction IVb, together with a photograph of a Coomassie blue-stained 4-15% gradient polyacrylamide gel showing the protein compositions of fractions in the eluate and indicating the fractions taken as Fraction Vb;

Figure 4 is a photograph of silver-stained 7.5% polyacrylamide gels showing the protein compositions of fractions from the eluate resulting from gel filtration of Fractions Va, Vb, Vc, and Vd from the purification of M. kandleri topoisomerase;

Figure 5A is a graph of the A_{280} (-------) and topoisomerase V activity (---o---) of the eluate from gel filtration of fraction Va;

Figure 5B is a photograph of a silver-stained 7.5% polyacrylamide gel showing the protein composition of fractions from the eluate resulting from gel filtration of fraction Va, showing additional fractions;

Figure 6 is an immunoblot of human topoisomerase II, λ Int protein, wheat germ topoisomerase I, calf thymus topoisomerase I, human topoisomerase I, and M. kandleri topoisomerase V with antibody to human topoisomerase I;

Figure 7 is an immunoblot of human topoisomerase I, fraction 63 from the gel filtration of fraction Va (S-200a-63), fractions 59 and 67 from the gel filtration of fraction Vc (S-200c-59 and S-200c-67), and fraction 63 from the gel filtration of fraction Vb (S-200b-63);

Figure 8A is a photograph of an agarose gel showing the time course of the M. kandleri topoisomerase V activity, with positively or negatively supercoiled pBR322 DNA in 30 mM Tris-HCl, pH 8.0 (at 25°C), 1 M potassium glutamate, and 5 mM Na_2EDTA, with electrophoresis in the presence of 1.6 ìg/ml chloroquine;

Figure 8B is a photograph of an agarose gel showing the effect of temperature on the enzymatic activity of M. kandleri topoisomerase V with positively supercoiled pBR322 DNA as substrate in 30 mM Tris-HCl, pH 8.0 (at 25°C), 1 M potassium glutamate, and 5 mM Na_2EDTA;

Figure 8C is a photograph of an agarose gel showing the effect of temperature on the enzymatic activity of M. kandleri topoisomerase V with negatively supercoiled pBR322 DNA as substrate in 30 mM Tris-HCl, pH 8.0 (at 25°C), 1 M potassium glutamate, and 5 mM Na_2EDTA, with electrophoresis in the presence of 25 ìg/ml chloroquine;

Figure 9A is a photograph of an agarose gel showing the effect of an increasing concentration of potassium glutamate on the enzymatic activity of M. kandleri topoisomerase V with a mixture of negatively and positively supercoiled pBR322 DNA as substrate, at 88°C in 30 mM Tris-HCl, pH 8.0 (at 25°C), and 5 mM Na_2EDTA, with electrophoresis in the presence of 1.6 ìg/ml chloroquine;

Figure 9B is a photograph of an agarose gel showing the effect of an increasing concentration of potassium chloride on the enzymatic activity of M. kandleri topoisomerase V with a mixture of negatively and positively supercoiled pBR322 DNA as substrate, at 88°C in 30 mM Tris-HCl, pH 8.0 (at 25°C), 5 mM Na_2EDTA, with electrophoresis in the presence of 1.6 ìg/ml chloroquine;

Figure 9C is a photograph of an agarose gel showing the effect of an increasing concentration of sodium chloride on the enzymatic activity of M. kandleri topoisomerase V with a mixture of negatively and positively supercoiled pBR322 DNA as substrate, at 88°C in 30 mM Tris-HCl, pH 8.0 (at 25°C), 5 mM Na_2EDTA, with electrophoresis in the presence of 1.6 ìg/ml chloroquine;

Figure 10A is a photograph of an agarose gel showing the effect of an increasing concentration of potassium glutamate on the enzymatic activity of M. kandleri topoisomerase V with negatively supercoiled pBR322 DNA as substrate, at 80°C in 30 mM Tris-HCl, pH 8.0 (at 25°C), and 5 mM Na_2EDTA;

Figure 10B is a photograph of an agarose gel as in Figure 10A, except that 5 mM $MgCl_2$ is substituted for 5 mM Na_2EDTA;

Figure 10C is a photograph of an agarose gel showing the effect of an increasing concentration of potassium chloride on the enzymatic activity of M. kandleri topoisomerase V with negatively supercoiled pBR322 DNA as substrate, at 80°C in 30 mM Tris-HCl, pH 8.0 (at 25°C), and either 5 mM $MgCl_2$ or 5 mM Na_2EDTA;

Figure 10D is a photograph of an agarose gel showing the effect of an increasing concentration of sodium chloride on the enzymatic activity of M. kandleri topoisomerase V with negatively supercoiled pBR322 DNA as substrate, at 80°C in 30 mM Tris-HCl, pH 8.0 (at 25°C), and either 5 mM $MgCl_2$ or 5 mM Na_2EDTA;

Figure 11 is a photograph of an agarose gel showing the relaxation of a unique topoisomer by M. kandleri topoisomerase V, with native pUC19 DNA or its unique topoisomer as substrate, and with electrophoresis in the presence of 2 ìg/ml of chloroquine;

Figure 12 is a photograph of an agarose gel showing the effect of an increasing concentration of single-stranded ÚX174 DNA on the relaxation of positively

or negatively supercoiled pBR322 DNA by M. kandleri topoisomerase V at 90°C or 70°C;

Figure 13A is a photograph of an agarose gel showing the effect of an increasing concentration of sodium chloride on the activity of M. kandleri topoisomerase on relaxed pBR322 DNA at 80°C in the presence of 5 mM Na_2EDTA or 5 mM $MgCl_2$, demonstrating the unlinking activity of the enzyme;

Figure 13B is a photograph of an agarose gel showing the effect of an increasing concentration of sodium chloride on the activity of M. kandleri topoisomerase on relaxed pBR322 DNA at 95°C in the presence of 5 mM Na_2EDTA or 5 mM $MgCl_2$, demonstrating the unlinking activity of the enzyme;

Figure 13C is a photograph of an agarose gel showing the effect of an increasing concentration of sodium chloride on the unlinking activity of M. kandleri topoisomerase on relaxed pBR322 DNA at 80°C in the presence of 5 mM Na_2EDTA or 5 mM $MgCl_2$, at higher concentrations of sodium chloride;

Figure 13D is a photograph of an agarose gel showing the effect of an increasing concentration of sodium chloride on the unlinking activity of M. kandleri topoisomerase on relaxed pBR322 DNA at 95°C in the presence of 5 mM Na_2EDTA or 5 mM $MgCl_2$, at higher concentrations of sodium chloride;

Figure 14A is a photograph of an two-dimensional agarose gel showing competition of M. kandleri topoisomerase V for substrate DNA with relaxed pBR322 DNA and relaxed pGEM3 DNA at low or high sodium chloride concentration, the electrophoresis being done without chloroquine in the first dimension and with 4 Ìg/ml chloroquine in the second dimension;

Figure 14B is a photograph of an two-dimensional agarose gel showing competition of M. kandleri topoisomerase V for substrate DNA with relaxed pBR322 DNA and composite preparation contained supercoiled and

relaxed pGEM3 DNA at low or high sodium chloride concentration, the electrophoresis being done without chloroquine in the first dimension and with 4 ìg/ml chloroquine in the second dimension;

Figure 15 is a photograph of an agarose gel showing the effect of dimethyl sulfoxide and camptothecin on the activity of M. kandleri topoisomerase V with pBR322 DNA as substrate at 70°C in 30 mM Tris-HCl, pH 8.0 (at 25°C), 1 M potassium glutamate, and 5 mM Na_2EDTA;

Figure 16 is a photograph of a silver-stained 4-15% gradient polyacrylamide gel and its autoradiograph showing covalent complex formation between pBR322 DNA and M. kandleri topoisomerase V;

Figure 17 is an autoradiograph of a denaturing 6% polyacrylamide gel showing pGEM1 DNA cleaved by M. kandleri topoisomerase V, indicating the site of cleavage;

Figure 18 shows the cleavage site of M. kandleri topoisomerase V on pGEM1 DNA in comparison to the consensus eukaryotic topoisomerase I site;

Figure 19 is a photograph of a two-dimensional agarose gel showing covalent complex formation between relaxed or supercoiled plasmid DNAs and M. kandleri topoisomerase V, the electrophoresis being done in the presence of 0.1% SDS;

Figure 20 is a photograph of a silver-stained 7.5%polyacrylamide gel of topoisomerase V fragments produced by the endoproteinase Lys-C under native conditions; and

Figure 21 is a photograph of a Coomassie blue-stained immunoblot of topoisomerase V fragments produced by the endoproteinase Lys-C under conditions of partial proteolysis and which have been used for protein microsequencing.

DESCRIPTION

DNA topoisomerase V has been purified from the hyperthermophilic methanogen Methanopyrus kandleri. M.

kandleri was isolated from a Guaymas Basin thermal vent submarine sediment sample taken by the research submersible Alvin (R. Huber et al., Nature 342:833-834 (1989)). It forms methane autolithotrophically using only H_2 and CO_2 as energy and carbon sources at temperatures up to 110°C, the upper temperature boundary at which living organisms have been found (K.O. Stetter, in Frontiers of Life (J. Tran Than Van et al., eds., Editions Frontières, Gif-sur-Yvette, 1992)).

The novel topoisomerase has properties similar to those of eukaryotic topoisomerase I, which distinguish it from all previously known prokaryotic topoisomerases. In particular, the enzyme is magnesium independent, it relaxes both negatively and positively supercoiled DNA, it makes a covalent complex with the 3' end of the broken DNA strand, it cleaves DNA at a site that is similar to the consensus sequence found for eukaryotic topoisomerases I, and it is recognized by antibody to human topoisomerase I. However, being remarkably thermostable and being able to unwind ccDNA, topoisomerase V differs from all known type 1-group B DNA topoisomerases.

I. PURIFICATION OF TOPOISOMERASE V

DNA topoisomerase V from M. kandleri can be purified by the following procedure: (1) lysis of the cells; (2) precipitation with polyethyleneimine and separation of the supernatant; (3) precipitation with ammonium sulfate; (4) phosphocellulose chromatography; (5) chromatography on heparin; and (6) gel filtration.

The product of this purification scheme is substantially purified M. kandleri DNA topoisomerase V substantially free of other enzymes acting on DNA.

A. **Lysis**

A suitable strain of M. kandleri is AV-19, DSM 24, Braunschweig, Germany. Lysis of the cells can be performed by methods generally known in the art that preserve enzymatic activity and minimize protein degradation. Preferably, lysis is carried out at about 4°C in the presence of at least one inhibitor of proteolytic activity. Lysis is preferably carried out in a disrupting device such as a French pressure cell.

A preferable lysis buffer contains 100 mM Tris-HCl, pH 8.0 at 25°C, 0.5 M NaCl, 10 mM ß-mercaptoethanol, 50 Ìg/ml of phenylmethylsulfonyl fluoride (PMSF), 50 Ìg/ml of N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 50 Ìg/ml of N-Á-p-tosyl-L-lysine chloromethyl ketone (TLCK), 50 Ìg/ml of pepstatin A, 50 Ìg/ml of leupeptin, and 1 mM benzamidine.

Typically, a quantity of cells is thawed in a water bath at room temperature in 1 milliliter of lysis buffer per 2 grams of cells and passed through a French pressure cell at 16,000 psi to give a lysate.

B. **Polyethyleneimine Precipitation**

The next step is the precipitation of the lysate with polyethyleneimine (Polymin P) and the collection of the supernatant. Typically, the lysate is diluted with lysis buffer to a final volume five times the original volume of lysis buffer, and centrifuged for two hours at high speed (40,000 rpm in a Beckman Instruments (Fullerton, California) Ti-50 rotor) (144,600 xg). To the recovered solution, a 5% solution of polyethyleneimine (pH 7.0) is added to the supernatant to a final polyethyleneimine concentration of 0.3%. After mixing for about 30 minutes at 0°C, the solution is centrifuged at

12,000 rpm (23,750xg) and the supernatant saved. The supernatant includes the topoisomerase V.

C. Precipitation with Ammonium Sulfate

In the next step of purification, the supernatant from the polyethyleneimine precipitation step is precipitated with saturated ammonium sulfate. Typically, a volume of 4 M ammonium sulfate equal to about 0.9 times the volume of the polyethyleneimine supernatant is added with stirring, and then solid ammonium sulfate is added to 90% saturation. The precipitate is collected by centrifugation after decantation of the supernatant.

About half of the proteins of M. kandleri are in the precipitate. Substantially all of the topoisomerase V activity is found in the pellet.

D. Phosphocellulose Chromatography

The next step is phosphocellulose chromatography. The pellet from ammonium sulfate precipitation is redissolved in a suitable starting buffer for phosphocellulose chromatography. A preferable starting buffer is 0.2 M NaCl, 30 mM sodium phosphate, pH 7.4 at 25°C, 10 mM ß-mercaptoethanol, 10% glycerol, 25 Ìg/ml each of PMSF, TPCK, and TLCK, 5 Ìg/ml of pepstatin A, 1 Ìg/ml of leupeptin, and 1 mM benzamidine, referred to as Buffer A + 0.2 M NaCl.

The redissolved pellet is dialyzed against starting buffer and loaded onto the column. The column is washed with the starting buffer after loading. Topoisomerase V is eluded with a linear gradient running from 0.2 to 1.0 M NaCl of a buffer containing the other components of starting buffer, followed by a linear gradient from 1.0 - 2.0 M NaCl in the same buffer. Active

topoisomerase V elutes from 0.55 - 0.73 M NaCl (fraction IVa) and also from 0.73 - 1.45 M NaCl (fraction IVb).

The resulting fractions are dialyzed against buffer B (10 mM sodium phosphate, pH 7.4 at 25°C, 10% glycerol, 2 mM ß-mercaptoethanol) + 0.5 M NaCl, if necessary after concentration.

E. Heparin Chromatography

The next step is heparin chromatography of the active fractions from the phosphocellulose chromatographic step. Preferably, fractions IVa and IVb are chromatographed separately on heparin. The dialyzed fractions are loaded onto a heparin column equilibrated with buffer B+0.5 M Nacl. After washing with the starting buffer the enzyme is eluted with a linear gradient of from 0.5 to 1.5 M NaCl in buffer B and fractions are collected. Active topoisomerase V elutes from about 1.0 to about 1.25 M NaCl for fraction IVa and from 0.95 to 1.25 M NaCl for fraction IVb. Preferably, the eluted topoisomerase enzyme is concentrated by rechromatography on a smaller heparin column after decrease of the sodium chloride concentration to 0.5 M by dilution with buffer B without sodium chloride. The enzyme is then re-eluted from the smaller column.

F. Gel Filtration

The final step in the purification process is gel filtration on a column capable of resolving proteins in the molecular weight range of about 50,000 daltons to about 200,000 daltons. A preferable chromatographic column comprises a HiLoad 16/60 Superdex 200 PG column (Pharmacia Biotech, Piscataway, NJ). Typically, the concentrated topoisomerase from the heparin chromatographic step is passed through the column which

has been equilibrated with 10 mM sodium phosphate, pH 7.4 at 25°C, 1 M NaCl, 5% glycerol, 2 mM ß-mercaptoethanol.

Topoisomerase V from fraction IVa that has been subjected to heparin chromatography and gel filtration chromatography has a specific activity of approximately 2.0×10^6 units per milligram in an assay to determine ATP-independent and Mg^{2+}-independent relaxation of supercoiled pBR322 DNA. For the assay, 1 Ìl of topoisomerase V preparation is incubated with a 50:50 mixture of positively and negatively supercoiled pBR322 DNA (0.2 Ìg) and assay buffer (30 mM Tris-HCl, pH 8.0 at 25°C, 1 M potassium glutamate, and 5 mM Na_2EDTA) in a 10 Ìl reaction mixture at standard assay conditions of 88°C for 15 minutes. The reactions were terminated by cooling them to 0°C and adding SDS to 1%. For topoisomerase assay in crude extracts, the reactions, after termination by SDS, were treated with 400 Ìg/ml of proteinase K at 37°C for one hour and then heated at 80°C for two minutes. Topoisomerization products are analyzed by 1.5% agarose gel electrophoresis in the presence of 1.6 Ìg/ml chloroquine at 3 V/cm for 10 hours. One unit of activity was defined as the amount of enzyme required to relax 50% of form I pBR322 DNA (0.2 Ìg) under standard conditions.

SDS polyacrylamide gel electrophoresis of the peak resulting from heparin chromatography and gel filtration of phosphocellulose peak IVa indicates that only one major protein peak is present. This protein has a molecular mass of 110,000 daltons as determined by SDS polyacrylamide gel electrophoresis, although its behavior on gel filtration chromatography corresponds to that of a globular protein with a molecular weight of about 142,000 daltons. Such inconsistencies between molecular weights obtained by polyacrylamide gel electrophoresis and gel filtration are not uncommon and reflect a degree of molecular asymmetry in the protein.

The gel filtration product represents approximately a 3,620-fold purification. If this is taken to represent purity, the heparin fraction is approximately 60% pure. However, in the context of this application, the term "substantially purified" means the following: (1) at least about a 40-fold purification with respect to the cleared lysate, and (2) substantially free of other enzymes acting on DNA. Such preparations are sufficiently pure to be useable in many of the applications of DNA topoisomerase V.

The purification is summarized in Table I.

TABLE I

PURIFICATION OF DNA TOPOISOMERASE V FROM *METHANOPYRUS KANDLERI*

Fraction	Step	Volume	Total Protein	Total Activity	Specific Activity	Yield
		ml	mg	units x 10^8	units/mg	%
I	Cleared lysate	259	7020	3.88	5.5×10^2	100
II	Polymin P	265	7580	5.30	7.0×10^2	136
III	Ammonium sulphate	438	3590	4.38	1.2×10^3	113
IVa	Phosphocellulose	135	90.0	2.16	2.4×10^4	55.6
Va	Heparin	13	0.767	0.91	1.2×10^6	23.4
VIa	Superdex 200	13	0.390	0.78	2.0×10^6	20.1
IVb	Phosphocellulose	100	186	1.50	8.1×10^3	38.6
Vb	Heparin	15	9.27	0.90	9.7×10^4	23.2
VIb	Superdex 200	13	0.563	0.10	1.9×10^5	2.7
VIc	Superdex 200	13	0.766	0.78	1.0×10^6	20.1
VId	Superdex 200	13	0.202	0.03	1.3×10^5	0.7
IV	Phosphocellulose	235	276	3.66	---	94.2
V	Heparin	28	10.0	1.81	---	46.6
VI	Superdex 200	52	1.92	1.69	---	43.5

II. PROPERTIES OF TOPOISOMERASE V

A. Enzymatic Activity

The topoisomerase V of the present invention is a type 1 topoisomerase. This enzyme has both relaxing and unlinking (or unwinding) activity on ccDNA as detailed below.

1. Topoisomerase V is a Type 1 Topoisomerase

Topoisomerase V from M. kandleri is a type 1 topoisomerase because it changes the linking number of a unique pUC19 DNA topoisomer in steps of one (Figure 11).

2. The Activity of DNA Topoisomerase V is Not Affected by Single-Stranded DNA

The activity of DNA topoisomerase V is not affected by the presence of single-stranded DNA. Figure 12 shows that the addition of single-stranded DNA ŰX174 DNA does not affect the relaxation of negatively or positively supercoiled pBR322 plasmid DNA by topoisomerase V at 70°C and 90°C at any ŰX174:pBR322 ratio up to 4.9:1. Lack of inhibition by single-stranded DNA distinguishes this enzyme from other thermophilic type 1 topoisomerases, topoisomerase I (reverse gyrase) and D. amylolyticus topoisomerase III which belong to group A and from mesophilic group B topoisomerases (J.J. Champoux in DNA Topology and Its Biological Effects (N.R. Cozzarelli and J.C. Wang, eds., Cold Spring Harbor Laboratory Press, New York, 1990), pp. 217-242).

From the inability of single-stranded DNA to inhibit topoisomerase V and from the ability of topoisomerase V to relax positively supercoiled DNA, Applicant believes that topoisomerase V requires double-

stranded regions of DNA for binding and topoisomerization, although Applicant does not intend to be bound by this theory and it is not required to account for the properties of the enzyme.

3. Ionic Conditions for Relaxation and Unlinking

The relaxing activity of topoisomerase V (occurs below the melting range of linear DNA) on supercoiled DNA in different ionic conditions of 80°C, 88°C, and 95°C is shown in Figures 9A-9C and 10A-10D and summarized in Table II. In Figures 9A-9C, a mixture of positively and negatively supercoiled DNA was used as the substrate and the results were analyzed by gel electrophoresis in the presence of chloroquine to separate positively from negatively supercoiled substrates. In Figures 10A-10D, a negatively supercoiled substrate was used and the products were analyzed by gel electrophoresis without chloroquine. In both Figures 9A-9C and Figure 10A-10D, the quantity of the enzyme was low enough to prevent complete relaxation and to monitor relative enzymatic activity by the extent of the conversion of the substrate to the product.

Complete relaxation of positively supercoiled DNA and practically complete relaxation of negatively supercoiled DNA by topoisomerase V is observed at 1.55 M potassium glutamate, 88°C, the condition considered optimal for DNA relaxation. Examples of the enzymatic activity include partial relaxation of supercoiled DNA, complete relaxation of a fraction of supercoiled DNA substrates, and complete relaxation of positively supercoiled DNA while having little activity on negatively supercoiled DNA. The enzyme can act distributively (i.e., the enzyme dissociates from the DNA after each catalytic cycle) or processively (i.e., the enzyme requires several catalytic cycles to occur before the enzyme dissociates).

The enzyme also prefers positively supercoiled over negatively supercoiled DNA, although it acts on both.

The substitution of chloride anion for glutamate anion substantially decreases the optimal salt concentration for relaxation from about 1.5 M to 0.5 M. The use of sodium cation instead of potassium decreases the optimum further by about 0.15 M so the optimum concentration of sodium chloride is about 0.30 to 0.35 M. The specific activity of the enzyme at optimal salt concentration is virtually salt-independent. There is virtually no effect of magnesium on relaxation by the enzyme, and EDTA does not inhibit the reaction. At 88°C, activity was detected at potassium glutamate concentrations of up to 3.1 M.

The ionic effects on the unlinking activity of topoisomerase V (the unlinking activity reveals itself in the melting range of linear DNA) differ from those on its relaxing activity. The data are shown in Figures 13A-13D. The unwinding of the topoisomers by topoisomerase V is more sensitive to high NaCl concentration: no unlinking occur at NaCl concentration as high as 0.5 M at 95°C, while the relaxing activity is still observed up to 0.65 M at 88°C. At 0.25-2.1 M potassium glutamate topoisomerase V uses the substrate DNA at 95°C for unlinking more efficiently than at 80 or 88°C for relaxation, if the extent of the conversion to the product is compared.

The data on the extent of the conversion to the product in the presence of $MgCl_2$ show that magnesium does not affect neither relaxation nor unlinking activities of topoisomerase V above 90 mM of monovalent salt. On the other hand, there is no effect of NaCl below 90 mM on the DNA unlinking at 95°C or DNA "re-relaxation" at 80°C in the presence of 5 mM $MgCl_2$. Applicant believes that it is likely due to the stabilizing effect of magnesium on the

DNA duplex, although Applicant does not intend to be bound by this theory.

TABLE II

EFFECT OF DIFFERENT IONS ON THE DNA RELAXATION BY *Mka* TOPOISOMERASE V

	Concentration (*M*) at which the activity is maximal			Highest concentration (*M*) at which the activity was detected		
	K-Glu	KCl	NaCl	K-Glu	KCl	NaCl
80°C, EDTA	1.5	0.45	0.30	2.1	0.55	0.45
80°C, $MgCl_2$	1.5	0.45	0.30	2.1	0.45	0.45
80°C, EDTA	1.5	0.50	0.35	3.1	0.65	0.65

4. **Reversibility of Topoisomerase V Binding to DNA**

At 300 mM NaCl, topoisomerase V freely dissociates and binds DNA (Figures 14A and 14B). This is shown by experiments in which the topoisomerase was prebound to one type of DNA and then a competitor DNA was added. The results of these experiments indicate that the topoisomerase V can dissociate from the type of DNA to which it was prebound and bind to another type of DNA. At lower ionic strength, the enzyme remains bound to the original substrate DNA, unlinking it, but does not bind significantly to competitor DNA. Remarkably, topoisomerase V unlinks DNA duplex at 50 mM NaCl in a highly processive mode without magnesium, by contrast to eukaryotic topoisomerase I which always requires magnesium for processivity (P.A. Der Garabedian <u>et. al.</u>, <u>Biochemistry</u> 30: 9940-9947 (1990)).

5. **Inhibitors of Topoisomerase V**

Camptothecin, which is a specific inhibitor of eukaryotic topoisomerase I from different species, has limited effect against the topoisomerase V of the present invention. However, dimethyl sulfoxide, which is used as a solvent for camptothecin and does not inhibit eukaryotic topoisomerases by itself, inhibits topoisomerase V (Fig. 15). This distinguishes the enzyme from eukaryotic topoisomerase I.

B. **Formation of Covalent Complex with DNA at 3'-End**

<u>M. kandleri</u> topoisomerase V covalently binds to the 3'-end of the DNA strand that it cleaves during the unlinking or unwinding reaction. Shorter DNA fragments appeared on a denaturing gel if 3'-labeled DNA was

incubated with topoisomerase V followed by the addition of protein denaturant (Fig. 17), but not if the DNA was labeled at the 5'-end. Thus, the enzyme covalently binds to the 3'-end of the broken DNA strand, preventing the 5'-labeled cleavable complex from entering the gel. The major topoisomerase V cleavage site (Fig. 18) on a 280-bp fragment of pGEM1 DNA has a sequence similar, but not identical, to the sequence of the consensus cleavage site for eukaryotic topoisomerase I (B.J. Bonven et. al., Cell 41: 541-551 (1985)).

If M. kandleri topoisomerase V is incubated with relaxed closed circular DNA, such as pBR322, pGEM3, or a derivative of pUC12 containing the consensus cleavage site for eukaryotic topoisomerase I and designated pHOT1, a covalent complex containing the topoisomerase and open circular DNA can be isolated (Example 12; Fig. 19)). The topoisomerase in this complex is covalently bound to the broken strand, as the complex is treated with protein denaturants such as sodium dodecyl sulfate.

Accordingly, the present invention also includes a complex comprising the topoisomerase of the present invention non-covalently bound to DNA, as well as a complex comprising the topoisomerase of the present invention covalently linked to the 3'-end of a DNA strand, which can be the 3'-end of a broken strand of open circular DNA.

C. **Antibody Reactivity**

M. kandleri topoisomerase V reacts with antibody raised against human topoisomerase I (Figs. 6-7). Human topoisomerase II and λ Int protein, which relaxes negatively and positively supercoiled DNA and binds covalently to the 3'-end of the broken DNA strand, do not react with antibody to human topoisomerase I.

The described properties of M. kandleri topoisomerase V enzyme identify it as a type 1-group B DNA topoisomerase (Table III). This is the first prokaryotic enzyme in this group.

Thus, a method of detecting and/or determining the M. kandleri topoisomerase V of the present invention can comprise the steps of:

(1) reacting the topoisomerase of the present invention with anti-human topoisomerase I antibody; and

(2) detecting and/or determining the topoisomerase V by detecting and/or determining an antigen-antibody complex between the topoisomerase and the antibody.

TABLE III

CLASSIFICATION OF TYPE 1 DNA TOPOISOMERASES

TYPE I DNA TOPOISOMERASES	GROUP A		
Location	bacteria, chloroplasts, yeast		
Covalent complex with the broken DNA strand	5'-P		
Substrate DNA for cleavage/religation	single-stranded		
Activity on positively supercoiled duplex DNA	-		
Mg^{2+} requirement for topoisomerization	+		
Strong cleavage sites	CNNN↑		other
ATP requirement for catalytic activity	+	-	-
Δlk per one cycle	+1 (pos. supercoiling)	± (relaxation)	± (relaxation)
Specific drugs	-	-	-
Examples	*Dam, Mka, Sac* topo I (rev. gyrase)	*Eco, Mlu* topo I	*Dam, Eco, Sce* topo III

TABLE III (continued)

CLASSIFICATION OF TYPE 1 DNA TOPOISOMERASES

TYPE I DNA TOPOISOMERASES	GROUP B		
Location	eukaryotic, nuclei, mitochondria, poxviruses *Methanopyrus kandleri*		
Covalent complex with the broken DNA strand	3'-P		
Substrate DNA for cleavage/religation	double-stranded		
Activity on positively supercoiled duplex DNA	+		
Mg^{2+} requirement for topoisomerization	-		
Strong cleavage sites	(C/T)CCTT↑	hexadecamer	
ATP requirement for catalytic activity	-	-	
Δlk per one cycle	± (relaxation)	± (relaxation)	
Specific drugs	-	-	camptothecin
Examples	VVir topo	*Mka* topo V	*Hsa, Sce, Tth* topo I

TABLE III (continued)

CLASSIFICATION OF TYPE 1 DNA TOPOISOMERASES

The consensus for DNA cleavage by *Eco* and *Dam* topoisomerases III has not been established and was shown to be different from CNNN↑, *Sce* topoisomerase III has a strong preference for ANN↑. Hexadecameric consensus for DNA cleavage by eukaryotic topoisomerases I: AGACIT↑AGA(A/G)AAA(A/I)(A/I)(A/I) was derived from the *Tetrahymena* rDNA repeats. *Dam*- *Desulfurococcus amylolyticus*, *Eco* - *Escherichia coli*, *HSA* - *Homo sapiens*, *Mka* - *Methanopyrus kandleri*, *Mlu* - *Micrococcus luteus*, *Sac* - *Sulfolobus acidocaldarius*, *Sce* - *Saccharomyces cerevisiae*, *Tth* - *Tetrahymena thermophilia*, VVir - Vaccinia virus

D. Partial Amino Acid Sequences

The amino-terminal amino acid sequence of M. kandleri DNA topoisomerase V is A-L-V-Y-D-A-E-F-V-G-S-E-R-E-F-E-E-E-R-E-T-F-L-K-G-V-K-A-Y-D-G-V-L-A-T-I-F-F-L. In this sequence, the amino acids occurring after the alanine (A) at amino acid 34 are somewhat uncertain.

Several proteolytic fragments of topoisomerase V have been generated with the proteolytic enzyme endoproteinase Lys-C (Promega, Madison, WI), which cleaves at the carboxylic side of lysine (K). Five such fragments have been generated: Fragment I, of about 50,000 daltons; Fragment II, a fragment of about 42,000 daltons; Fragment III, a fragment of about 36,000 daltons; Fragment IV, a fragment of about 33,000 daltons; and Fragment V, a fragment of about 25,000 daltons. Fragment III is from the carboxyl-terminus of the protein. In addition, fragments of about 75,000 daltons and 55,000 daltons (Fragment N1 and N2, respectively) can be isolated and, by its protein sequence, are from the amino-terminus of the enzyme (Example 13). Although Applicant does not intend to be bound by this theory, at least Fragment III and Fragment N1 are believed to represent stable domains within the enzyme and can be used as immunogens for the production of antibodies, including monoclonal antibody if desired. Such antibody is an aspect of the present invention.

The amino-terminal amino acid sequence of Fragment I is K-S-D-T-E-T-I-E-T.

The partial amino acid sequence of Fragment II is K-P-E-L-P-Y-V-A-V-P-P-H-M-A-E-R-A-R-R-V-L-T-R-E-D-D-L-A-X-D-V-X-A. In this sequence, "X" represents an amino acid that cannot be determined. In addition, the proline (P) at

amino acid 11, the threonine (T) at amino acid 22, and the arginine (R) at amino acid 23 are somewhat uncertain.

The partial amino acid sequence of Fragment III is K-R-V-P-R-A-X-X-G-X-X-F-D-R-L. In this sequence, "X" represents an amino acid that cannot be determined.

The partial amino acid sequence of Fragment IV is K-S-G-R-Q-E-R-S-E-E-E-E-K-E-E-L-E-R-K-V-G-E-G-R-A-R-R-L-I-E-Y-F-G-S-A.

The partial amino acid sequence of Fragment V is K-Y-G-S-A-S-X-X-R-R-L-P-X-E-E-X-R-E-L-G-F-X-D-D-R. In this sequence, "X" represents an amino acid that cannot be determined. In addition, the tyrosine (Y) at amino acid (2), the serine (S) at amino acid 6, the arginines (R) at residues 17 and 25, and the aspartic acid (D) at amino acid 24 are somewhat uncertain.

The availability of the amino-terminal amino acid sequence of <u>M. kandleri</u> topoisomerase V means that the present invention includes thermostable type 1-group B DNA topoisomerases possessing the amino-terminal amino acid sequence of <u>M. kandleri</u> topoisomerase V. In this context, the term "thermostable" is defined as possessing detectable relaxing activity at a temperature of 50°C or higher. Additionally, enzymes containing conservative amino acid substitutions within this sequence are also within the scope of the invention. Such conservative amino acid substitutions include, but are not limited to, substituting any of isoleucine (I), valine (V), and leucine (L) for any other of these amino acids; aspartic acid (D) for glutamic acid (E) and vice versa; glutamine (Q) for asparagine (N) and vice versa; and serine (S) for threonine (T) and vice versa. The above-mentioned substitutions are not the only amino acid substitutions that can be considered "conservative." Other

substitutions can also be considered conservative, depending on the environment of the particular amino acid. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can be alanine (A) and valine (V). Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine (L) and isoleucine (I), and sometimes with valine (V). Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the differing pK's of these two amino acid residues are not significant. Still other changes can be considered "conservative" in particular environments.

The present invention also includes thermostable type 1-group B DNA topoisomerases possessing at least one of the following amino acid sequences that is from Fragment I, Fragment II, Fragment III, Fragment IV, and Fragment V and is unequivocally known:

(1) K-S-D-T-E-T-I-E-T (from Fragment I);

(2) K-P-E-L-P-Y-V-A-V-P-P-H-M-A-E-R-A-R-R-V-L-T-R-E-D-D-L-A-X-D-V-X-A (from Fragment II);

(3) K-R-V-P-R-A-X-X-G-X-X-F-D-R-L (from Fragment III);

(4) K-S-G-R-Q-E-R-S-E-E-E-E-K-E-E-L-E-R-K-V-G-E-G-R-A-R-R-L-I-E-Y-F-G-S-A (from Fragment IV); or

(5) K-Y-G-S-A-S-X-X-R-R-L-P-X-E-E-X-R-E-L-G-F-X-D-D-R (from Fragment V).

The invention further includes thermostable type 1-group B DNA topoisomerases possessing at least one amino acid sequence related to sequence (1), (2), (3), (4) or (5) by one or more conservative amino acid substitutions.

Also within the scope of the present invention are modifications of these enzymes, including, but not limited to, enzymes bound to solid supports.

III. USE OF TOPOISOMERASE ACCORDING TO THE PRESENT INVENTION

Topoisomerase V according to the present invention can be used, as described above, either to relax supercoiled DNA or to unlink ccDNA.

In general, the unlinking reaction comprises treating a ccDNA with the enzyme of the present invention at a temperature at least as high as the melting range of the linear form of treated DNA and ionic conditions that allow the enzyme to bind to DNA and catalyze the unlinking reaction; the enzyme binds to DNA and catalyzes the unlinking reaction, driven by DNA melting, and ccDNA with Lk lower than the Lk of the DNA before treatment is produced.

This reaction is equally applicable to any procedure that involves primer annealing and/or elongation, i.e., PCR, LCR, hybridization probe preparation, including DNA template preparation for hybridization of large probes.

The relaxation reaction comprises treating a supercoiled DNA with the enzyme of the present invention at a temperature below the melting range of linear form of the treated ccDNA and ionic conditions that allow the enzyme to bind to DNA and catalyze the relaxation reaction to produce at least partially relaxed DNA. The DNA to be relaxed can be positively supercoiled DNA, negatively supercoiled DNA, or a mixture of the two types of supercoiled DNA.

These relaxation and unlinking reactions are particularly useful for manipulation of DNA conformation, for preparation of ccDNA with desired degree of

supercoiling using only one enzyme and varying only ionic conditions and temperature.

A unique salt tolerance of the enzyme makes it an indispensable tool in chromatin reconstitution in vitro.

Topoisomerase V can be used to form a covalent complex with DNA. The method of covalent complex formation comprises:

(1) incubating the topoisomerase of the present invention with DNA under ionic conditions allowing the binding of the topoisomerase to the DNA and the breaking of one strand by the topoisomerase; and

(2) denaturing of the topoisomerase leaving a covalent complex between the topoisomerase and the DNA.

Topoisomerase V can also be used as a specific endonuclease. The method of use of topoisomerase V as a specific endonuclease comprises:

(1) incubating the topoisomerase of the present invention with DNA under ionic conditions allowing the binding of the topoisomerase to the DNA and the breaking of one strand by the topoisomerase at a specific sequence; and

(2) then denaturing the topoisomerase yielding cleaved DNA.

The sequence at which cleavage occurs, as shown in Figure 18, is related to the site of cleavage by eukaryotic topoisomerase I enzymes.

Additionally, topoisomerase V can be used to produce an activated DNA substrate with at least a single amino acid residue covalently bound to the 3'-PO_4 terminus of a DNA strand adjacent to the recognition site of the topoisomerase of the present invention. The process comprises:

(1) incubating the topoisomerase with DNA under ionic conditions allowing the binding of the topoisomerase to DNA and the breaking of one strand by the topoisomerase at a specific sequence; and

(2) then denaturing the topoisomerase and hydrolyzing the topoisomerase with a nonspecific protease.

The purified protein can also be used to prepare antibodies, both polyclonal antibodies and monoclonal antibodies. A protein of molecular weight about 110,000 is sufficiently large that it does not need to be coupled to a carrier such as keyhole limpet hemocyanin to be immunogenic. Methods for the preparation of polyclonal antibodies are well-known in the art and need not be described in detail. Such methods are disclosed, for example, in E. Harlow & D. Lane, Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988), pp. 53-137, incorporated herein by this reference.

Once polyclonal antibodies are produced, antibody-producing cells can be fused with appropriate melanoma cells to form hybridomas in order to produce monoclonal antibodies. Such monoclonal antibodies can be produced by techniques well-known in the art; typically, developments and adaptations of the basic technique of G. Köhler & C. Milstein, "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity", Nature 256: 495-497 (1975), incorporated herein by this reference, are used. Such developments and adaptations are described, for example, in Harlow & Lane, Antibodies: A Laboratory Manual, supra, pp. 139-281, incorporated herein by this reference.

EXAMPLES

The invention is illustrated by the following Examples. These examples are for exemplification purposes only and do not limit the invention.

Example 1
Purification of DNA Topoisomerase V from Methanopyrus kandleri

Materials and Methods

Methanopyrus kandleri strain AV-19, DSM 6324, Braunschweig, Germany was used. The cells were grown in BSM medium at 100°C in a 300-liter enamel protected fermentor (HTE Bioengineering, Wald, Switzerland) with stirring and continuous gassing (H_2/CO_2, 80:20). Exponentially growing cells were rapidly cooled and harvested with a separator (Westfalia, Germany). The cells were stored at -70°C.

Protease inhibitors phenylmethylsulfonyl fluoride (PMSF), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), N-Á-p-tosyl-L-lysine chloromethyl ketone (TLCK), pepstatin A, leupeptin, and benzamidine were from Calbiochem (La Jolla, CA). ß-mercaptoethanol was from Fisher (Pittsburgh, PA), polyethyleneimine (Polymin P) was from Sigma (St. Louis, MO), ammonium sulfate was from BRL (Gaithersburg, MD), and glycerol was from Mallinckrodt (Chesterfield, MO). Phosphocellulose P11 was from Whatman (Clifton, NJ). HiTrap Heparin columns (1 ml and 5 ml volume), and HiLoad 16/60 Superdex 200 PG gel filtration columns were from Pharmacia LKB (Piscataway, NJ). Centriprep 30 concentration cartridges were from Amicon (Beverly, MA).

Methods of Protein Characterization

Protein concentrations were determined spectrophotometrically. Protein compositions of the fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on Bio-Rad (Richmond, CA) Mini-Protein II according to the method of Laemmli or using Bio-Rad precast gels. The gels were stained with silver using the Bio-Rad silver stain kit or with Coomassie G-250 brilliant blue according to the method of Merril (C.R. Merril in <u>Protein Purification</u> (M.P. Deutscher, ed., Academic Press, San Diego, 1990), pp. 685-687)).

Topoisomerase Assay

Topoisomerase V activity was assayed by ATP-independent and Mg^{2+}-independent relaxation of supercoiled pBR 322 DNA. An aliquot (1 ìl) of topoisomerase V preparation was incubated with a 0.2ìg of a 50:50 mixture of positively and negatively supercoiled pBR322 DNA in "standard" buffer (30 mM Tris-HCl, pH 8.0 at 25°C, 1 M potassium glutamate, and 5 mM Na_2EDTA) in a 10- ìl reaction mixture at "standard" conditions of 88°C for 15 minutes. The reactions were terminated by rapidly cooling to 0°C and adding sodium dodecyl sulfate to 1% concentration. For topoisomerase assay in crude extracts, the reactions, terminated by SDS, were treated with proteinase K (400 ìg/ml at 37°C for one hour) and then heated at 80°C for two minutes. The topoisomerization products were analyzed by 1.5% agarose gel electrophoresis in the presence of 1.6 ìg/ml chloroquine at 3 V/cm for ten hours. One unit of activity was defined as the amount of enzyme required to relax 50% of form I pBR322 DNA (0.2 ìg) in standard buffer in 15 minutes at 88°C.

Protein Purification

All steps were performed at 4°C unless indicated. Chromatography was done on a LKB liquid chromatography system composed of a HPLC pump, a variable wavelength monitor reading at 280 nm, a controller, a 2-channel recorder, and a rack.

M. kandleri cells (120 g wet weight) were thawed in a water bath at room temperature in 60 ml lysis buffer (100 mM Tris-HCl, pH 8.0 at 25°C, 0.5 M NaCl, 10 mM ß-mercaptoethanol, 50 ìg/ml each of PMSF, TPCK, TLCK, pepstatin A, and leupeptin, and 1 mM benzamidine) and passed through a French pressure cell (American Instrument (Baltimore, MD) at 16,000 psi. The recovered solution was diluted with lysis buffer to a final volume of 300 ml and centrifuged for two hours at 40,000 rpm in a Beckman Instruments (Fullerton, CA) Ti-50 rotor (fraction I, 259 ml). A 5% solution of polyethyleneimine (Polymin P) (pH 7.0) was added dropwise to the supernatant with constant stirring to a final concentration of 0.3%. After mixing for 30 minutes at 0°C, the solution was centrifuged at 2,000 rpm for 40 minutes in a Sorvall RC-5B centrifuge. The supernatant was saved (fraction II, 265 ml) and 245 ml of 4 M ammonium sulfate was added under stirring. Then ammonium sulfate was added to 90% saturation and the solution was left in a cold room overnight with stirring. Not all of the ammonium sulfate dissolved. The supernatant (545 ml) was decanted and centrifuged at 11,000 rpm in a Sorvall RC-5B centrifuge for two hours.

The pellet from the ammonium sulfate precipitation was dissolved in 400 ml starting buffer for phosphocellulose chromatography (Buffer A + 0.2 M NaCl). Buffer A is 30 mM sodium phosphate, pH 7.4 at 25°C, 10 mM ß-mercaptoethanol, 10% glycerol, 25 ìm each of PMSF, TPCK, and TLCK, 5 ìg/ml pepstatin A, 1 ìg/ml leupeptin, and 1 mM

benzamidine. The dissolved pellet was dialyzed against two liters of the same buffer with two changes (fraction III, 438 ml). After centrifugation, the solution was loaded onto a phosphocellulose P11 column (Whatman) (2.6 x 30 cm) equilibrated with buffer A + 0.2 M NaCl. After loading, the column was washed with three volumes of buffer A + 0.2 M NaCl. Topoisomerase V was eluted with a 600-minute linear gradient of 0.2 to 1.0 M NaCl in Buffer A at 1.5 ml/min, followed by a 400-minute linear gradient of 1.0 to 2.0 M NaCl in Buffer A. Fractions of 15 ml were collected and assayed for DNA relaxation activity. Active fractions were combined into two pools: A first pool of 0.55-0.73 M NaCl (135 ml) and a second pool of 0.73-1.45 M NaCl (620 ml). The latter was concentrated in Centriprep 30 cartridges to a final volume of 100 ml.

Figure 1 shows the results of phosphocellulose chromatography. The A_{280} and NaCl concentration for the eluate from the phosphocellulose column are shown, together with a photograph of a silver-stained 4-15% SDS-polyacrylamide gel showing the protein compositions of fractions in the eluate and indicating the fractions taken for further purification as Fractions IVa and IVb. For the polyacrylamide gel, 5 Ìl (fractions 29-41) or 20 Ìl (fractions 51-75) were electrophoresed.

Both pools were dialyzed against Buffer B plus 0.5 M NaCl. Buffer B was 10 mM sodium phosphate, pH 7.4 at 25°C, 10% glycerol, 2 mM ß-mercaptoethanol (Fraction IVa, 135 ml, and fraction IVb, 100 ml respectively.)

Fraction IVa was loaded onto a 5-ml HiTrap heparin column, equilibrated with buffer B plus 0.5 M NaCl. After the column was washed with 5 volumes of buffer B plus 0.5 M NaCl, a 100-minute linear gradient of 0.5-1.5 M NaCl in buffer B at 0.5 ml/min was applied.

Figure 2 shows the results of heparin chromatography of Fraction IVa. The A_{280} and NaCl concentration are shown, along with a photograph of a Coomassie blue-stained 4-15% gradient SDS-polyacrylamide gel showing the protein compositions of fractions in the eluate and indicating the fractions taken as Fraction Va. For the polyacrylamide gel, 5-Ìl aliquots of the fractions were electrophoresed. Topoisomerase was a major protein band in fractions 30 and 32.

Active fractions (1 ml) between 1.0-1.25 M NaCl were pooled (fraction Va, 13 ml), and concentrated on a 1-ml HiTrap heparin column by decreasing the sodium chloride concentration to 0.5 M by dilution with buffer B without sodium chloride, loading the sample on the smaller column and re-eluting with the linear gradient of NaCl. The concentrate was passed through a HiLoad 16/60 Superdex 200 PG column equilibrated with 10 ml sodium phosphate, pH 7.4 at 25°C, 1 M NaCl, 5% glycerol, 2 mM ß-mercaptoethanol (fraction Va, 13 ml).

Fraction IVb was chromatographed on a 5-ml HiTrap heparin column in the same way as Fraction IVa, with the results being shown in Figure 3. The active fractions eluted between 0.95-1.25 M NaCl. These fractions were combined (fraction Vb, 15 ml) and concentrated on a 1-ml HiTrap heparin column as above. 90% of the total activity which eluted between 1.07-1.17 M NaCl was combined into 3 separate pools of 3, 2, and 3 ml, respectively. Each of the pools was subjected to gel filtration as above. This resulted in fractions VIb, VIc, and VId, of 13 ml each. Fractions VIa-VId were stored at 4°C or -80°C.

The protein compositions of fractions from the eluates resulting from gel filtration of Fractions Va, Vb, Vc, and Vd are shown in Figure 4. In Figure 4, 5 Ìl (for

Va, Vb, and Vc) or 10 ìl was electrophoresed on a 7.5% polyacrylamide gel and stained by silver.

Figure 5A shows the gel filtration profile of Fraction Va in terms of A_{280} (-------) and enzymatic activity (----o----).

Figure 5B shows the protein composition of fractions from this gel filtration eluate, as in Figure 4, with additional fractions shown.

The purification is summarized in Table I. Overall purification, as determined by the fraction with the highest specific activity, fraction VIa, was 3620-fold.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of fraction VIa (Figs. 4 and 5B) shows that the only significant protein component in this fraction has a molecular weight of about 110,000. This is to be compared with the apparent molecular weight on gel filtration of about 142,000 (Fig. 5A). Such discrepancies are not uncommon, and arise from molecular asymmetry of the protein. The gel filtration molecular weight indicates that the enzyme occurs as a monomer.

Heparin chromatography of fraction IVb resulted in a 12-fold purification. The protein composition of this fraction, however, remained complex (Fig. 3). Collection of the heparin eluate into three pools and application of each pool separately on a Superdex 200 gel filtration column resulted in preparations giving a protein peak in the position where pure topoisomerase V eluted (Fig. 4). In all three cases, topoisomerase activity was centered around this peak. The active fractions also contain an unknown 75,000-dalton protein (Fractions VIb and VId) or both a 75,000-dalton and an

80,000-dalton protein (Fraction VIc) along with topoisomerase V (Fig. 4). Fraction VIc, which contains as much enzymatic activity as Fraction VIa does, was purified 10-fold in the gel filtration step to about 50% purity.

Example 2

Recognition of M. kandleri Topoisomerase V by Antibody to Human Topoisomerase I

Polyclonal antibody to human topoisomerase I binds M. kandleri topoisomerase V as determined by Western blotting. In Figure 6, human topoisomerase II (Topogen, Columbus, OH, 10 Ìl, 20 units) (lane 1), λ Int protein (220 ng) (lane 2), wheat germ topoisomerase I (Promega, Madison, WI) (5 Ìl, 50 units) (lane 3), thymus topoisomerase I (United States Biochemical, Cleveland, OH) (5 Ìl, 50 units) (lane 4), human topoisomerase I (Topogen)) (5 Ìl, 25 units) (lane 5), and M. kandleri topoisomerase V (200 ng (lane 6) and 50 ng (lane 7)) were electrophoresed on a 7.5% SDS-polyacrylamide gel and blotted onto Immobilon P membrane (Millipore, Bedford, MA) for Western blotting. The blot was blocked with 5% dry milk in phosphate buffered saline (PBS) and probed with rabbit anti-human topoisomerase I antibody (Dr. L. Liu, Johns Hopkins University, Baltimore, MD) diluted 1:500. Immunoreactive material was detected with [^{125}I] protein A (NEN, Boston, MA), and processed by a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). This Western blot indicates that the antibody recognized only human topoisomerase I, wheat germ topoisomerase I and M. kandleri topoisomerase V. A breakdown product of human topoisomerase I, with a molecular weight of about 66,200 daltons was also recognized.

A similar Western blot was performed on individual fractions of the eluate from the gel filtration

column for Fractions VIa, VIb, and VIc. (Fig. 7). In Figure 7, 50 units of human topoisomerase I (lane 1), 2 ìl of gel filtration fraction 63 from Fraction VIa (lane 2), 5 ìl of gel filtration fractions 59 and 67 from Fraction VIc (lanes 3 and 4), and 5 ìl of gel filtration fraction 63 from Fraction VIb (lane 5) were used. These results show that the smaller proteins occurring in Fractions VIb and VIc did not react with antibody human topoisomerase I, unlike the breakdown product of human topoisomerase I itself. This indicates that the 75,000-dalton and 80,000-dalton proteins are not breakdown products of topoisomerase V.

Example 3
Time Course and Temperature Dependence of Topoisomerase V Activity

The time course and temperature dependence of topoisomerase V activity were determined by incubating positively or negatively supercoiled pBR322 DNA with 2 ng or 20 ng topoisomerase V for various lengths of time (Fig. 8A) or at different temperatures (Figs.8B and 8C).

In Figure 8A, positively supercoiled (lanes 1-5) or negatively supercoiled (lanes 6-10) pBR322 DNA was incubated with 2 ng of M. kandleri topoisomerase V under standard assay conditions for the indicated times. Gel electrophoresis was carried out in the presence of 1.6 ìg/ml chloroquine, so that the relaxation of both positively and negatively supercoiled DNA results in the decrease of mobility of topoisomers.

In Figure 8B, positively supercoiled pBR322 DNA was incubated with 20 ng of topoisomerase V at the indicated temperatures, and gel electrophoresis was carried out without chloroquine. Completely relaxed

pBR322 DNA ("rel") during incubation at 70-90°C is slightly negatively supercoiled.

In Figure 8C, negatively supercoiled pBR322 was incubated with 20 ng of topoisomerase V at the indicated temperatures, and gel electrophoresis was carried out with 25 ìg/ml of chloroquine. The negatively supercoiled substrate DNA runs as slightly positively supercoiled on the gel. The product topoisomers at 94°C had a Lk lower than the substrate DNA, while at 100°C, the products ("U") had Lk substantially less than that of substrate, the topoisomers remaining highly unwound even in the presence of 25 ìg/ml of chloroquine.

The purified enzyme relaxes both positively and negatively supercoiled DNA with nearly equal proficiency. The final distribution of topoisomers does not depend on the initial supercoiling, and is not affected by a severalfold change in enzyme:DNA ratio. The relaxation activity is catalytic and processive. The rate of DNA relaxation is proportional to the quantity of enzyme added and is about 15 supercoils per minute per enzyme monomer. At 70-90°C the enzymatic reaction yields fully relaxed duplex DNA. At temperatures above 90°C the enzyme produces highly unlinked forms of DNA. This effect, named unlinking, i.e., a substantial reduction in DNA linking number, is caused by DNA melting.

Example 4

Effect of Ions on Activity of Topoisomerase V

DNA topoisomerase V does not require specific ions for activity. It relaxes DNA in NaCl, KCl, and potassium glutamate. (Figs. 9A-9C, 10A-10D). The optimum condition for relaxation is 1.55 M potassium glutamate. EDTA, and Mg^{2+} do not interfere with the relaxation activity.

Figure 9A is a photograph of an agarose gel showing the effect of an increasing concentration of potassium glutamate on the enzymatic activity of topoisomerase V (1.5 units) with a mixture of negatively and positively supercoiled pBR322 DNA (0.1 Ìg each form) as substrate, at 88°C in 30 mM Tris-HCl, pH 8.0 at 25°C, and 5 mM Na_2EDTA. The potassium glutamate concentration is as indicated. Incubations were carried out for 15 minutes, as is the case for all other assays shown unless indicated. Electrophoresis was in the presence of 1.6 Ìg/ml chloroquine. Lane 1 is a control.

Figure 9B is a photograph of an agarose gel showing the effect of an increasing concentration of potassium chloride on the enzymatic activity of topoisomerase V (1.5 units) with a mixture of negatively and positively supercoiled pBR322 DNA (0.1 Ìg each form) as substrate, at 88°C in 30 mM Tris-HCl, pH 8.0 at 25°C, and 5 mM Na_2EDTA. The potassium chloride concentration is as indicated. Electrophoresis was in the presence of 1.6 Ìg/ml chloroquine. Lane 1 is a control.

Figure 9C is a photograph of an agarose gel showing the effect of an increasing concentration of sodium chloride on the enzymatic activity of topoisomerase V (1.5 units) with a mixture of negatively and positively supercoiled pBR322 DNA (0.1 Ìg each form) as substrate, at 88°C in 30 mM Tris-HCl, pH 8.0 at 25°C, and 5 mM Na_2EDTA. The sodium chloride concentration was as indicated. Electrophoresis was in the presence of 1.6 Ìg/ml chloroquine. Lane 1 is a control.

Complete relaxation of positively supercoiled DNA and practically complete relaxation of negatively supercoiled DNA to topoisomerase V was achieved at 1.55 M potassium glutamate at 88°C.

Figures 10A-10D show the effect of varying ionic environments on the activity of topoisomerase V on negatively supercoiled DNA as substrate.

Figure 10A is a photograph of an agarose gel showing the effect of an increasing concentration of potassium glutamate on the enzymatic activity of topoisomerase V (3 units) with negatively supercoiled pBR322 DNA (0.2 ìg; "-sc") as substrate, at 80°C in 30 mM Tris-HCl, pH 8.0 at 25°C, and 5 mM Na_2EDTA. Lane 1 is a control. Electrophoresis was without chloroquine.

Figure 10B is a photograph of an agarose gel as in Figure 10A, except that 5 mM $MgCl_2$ was substituted for 5 mM Na_2EDTA. Lane 1 is a control.

Figure 10C is a photograph of an agarose gel showing the effect of an increasing concentration of potassium chloride on the enzymatic activity of topoisomerase V (3 units) with negatively supercoiled pBR322 DNA (0.2 ìg) as substrate, at 80°C in 30 mM Tris-HCl, pH 8.0 at 25°C, and either 5 mM $MgCl_2$ (lanes 1-8) or 5 mM Na_2EDTA (lanes 9-16).

Figure 10D is a photograph of an agarose gel as in Figure 10C, except that sodium chloride was substituted for potassium chloride.

These results indicate that Cl^- anion substantially decreases the optimal salt concentration for DNA relaxation by topoisomerase V. Also, the use of Na^+ cation instead of K^+ decreases the optimum. There is a tendency, however, for the optimum NaCl and KCl concentrations to increase with increasing temperature.

Example 5

M. kandleri Topoisomerase V is a Type 1 Topoisomerase

M. kandleri topoisomerase V is a type 1 topoisomerase. The enzyme changes the linking number of a unique pUC19 DNA topoisomer in steps of 1 (Fig. 11).

Figure 11 is a photograph of an agarose gel showing the relaxation of a unique topoisomer by topoisomerase V, with 0.2 Ìg of native pUC19 DNA (lanes 1 and 2) or its unique topoisomer (lanes 3 and 4) as substrate and 15 units of enzyme (lanes 2 and 4), with electrophoresis in the presence of 2 Ìg/ml of chloroquine.

The results of Figure 11 indicate that a number of topoisomers are detectable as a result of the action of topoisomerase V on a unique negatively supercoiled topoisomer of pUC19 DNA. The Lk of the produced topoisomers increases in steps of 1.

Example 6

Single-Stranded DNA Does Not Affect the Relaxation of Negatively or Positively Supercoiled DNA by M. kandleri Topoisomerase V

Single-stranded ÚX174 DNA does not affect the relaxation of negatively or positively supercoiled pBR322 DNA by topoisomerase V at 70°C and 90°C at any ÚX:pBR 322 ratio up to 4.9 (Fig. 12).

Figure 12 is a photograph of an agarose gel showing the effect of an increasing concentration of single-stranded ÚX174 DNA on the relaxation of positively (0.2 Ìg; lanes 2-6) or negatively (0.2 Ìg; lanes 7-11) supercoiled pBR322 DNA by topoisomerase V (5 units) at 90°C (panel A) or 70°C (panel B). Controls were positively supercoiled pBR322 DNA (lane 1), negatively

supercoiled pBR322 DNA (lane 12), single-stranded ÚX174 DNA before incubation (lane 13), and single-stranded ÚX174 DNA after incubation (lane 14).

This data suggests that topoisomerase V enzyme does not form an irreversible complex between the enzyme and short duplex regions of single-stranded ÚX174 DNA, unlike mesophilic eukaryotic topoisomerase I enzymes, which are inhibited by short duplex regions formed in single-stranded ÚX174 DNA. These results also indicate that topoisomerase V requires double-stranded regions of DNA for binding and topoisomerization.

Example 7

Unlinking Activity of Topoisomerase V

Topoisomerase V from M. kandleri catalyzes an unlinking reaction; that is, a reaction which decreases Lk of closed circular DNA below its initial value. This is indicative of at least partial unwinding of the Watson-Crick double helical structure. Unlinking activity is favored at higher temperature: the higher the temperature, the more unwound DNA is generated (Figs. 8B,C)

The unlinking activity of topoisomerase V is most clearly demonstrated by activity on relaxed DNA (Figs. 13A-13D). This is because the products are clearly distinguishable from the substrate on gel electrophoresis, as the unlinking causes an increased mobility of the relaxed DNA.

Figure 13A is a photograph of an agarose gel showing the effect of an increasing concentration of sodium chloride on the activity of topoisomerase V (2 units) on relaxed pBR322 DNA (0.2 ìg) at 80°C in the presence of 5 mM Na_2EDTA (lanes 2-7) or 5 mM $MgCl_2$ (lanes 9-14). Lanes 1 and 8 contained no enzyme.

Figure 13B is a photograph of an agarose gel as in Figure 13A, but with the enzymatic reaction having been performed at 95°C.

Figure 13C is a photograph of an agarose gel as in Figure 13A, but at higher concentrations of sodium chloride and with a greater quantity (10 units) of enzyme. Lanes 5 and 10 contain no enzyme. Lanes 1-5 contain 5 mM Na_2EDTA; lanes 6-10 contain 5 mM $MgCl_2$.

Figure 13D is a photograph of an agarose gel as in Figure 13C, but with the enzymatic reaction having been performed at 95°C.

Similar results were obtained with an increasing concentration of potassium glutamate on the degree of conversion of the initial substrate (a mixture of negatively and positively supercoiled pBR322 DNA (0.1 ìg each form)) into highly unwound form of ccDNA by topoisomerase V (1.5 units) at 95°C in 30 mM Tris-HCl, pH 8.0 at 25°C, 5 mM Na_2EDTA.

At 75 and 150 mM of monovalent cation, the products of the unlinking reaction can be seen as the band between +sc and -sc bands in Figures 9A, 9B, and 9C in lanes 2 and 3. At constant temperature, an increase in ionic strength of the reaction mixture results in a switch from the unlinking mode to the relaxation mode of activity.

Topoisomerase V retains its unlinking activity at NaCl concentrations as low as 1 mM. The lower the ionic strength, the lower the temperature at which unlinking occurs (Fig. 13A, lane 2; Fig. 13B, lane 9). When the unlinking reaction is performed at 95°C, intermediate topoisomers are seen at 155 and 191 mM NaCl and are absent

at salt concentrations from 1 mM NaCl to 91 mM NaCl (Figs. 13B, 13D). This suggests the unlinking of relaxed DNA is processive at low salt concentrations and tends to be distributive at high salt concentrations.

Example 8

Reversibility of <u>M. kandleri</u> Topoisomerase V Binding to DNA

At high ionic strengths, topoisomerase V readily dissociates from one DNA molecule and binds another, acting on both DNA molecules. However, at lower ionic strength, topoisomerase V is tightly bound to the DNA molecule to which it binds first and disassociation and binding to another DNA molecule is inefficient. These results are shown in Figures 14A and 14B.

In these experiments, topoisomerase V was preincubated with substrate DNA at high salt (300 mM NaCl). Then the sample was diluted and the NaCl concentration was adjusted either to 300 mM NaCl or low salt (50 mM). Competitor DNA was then added. The enzymatic reaction was performed at 90°C and the results were analyzed by two-dimensional gel electrophoresis.

Figure 14A is a photograph of an two-dimensional agarose gel showing competition of topoisomerase V (100 units) for substrate DNA with relaxed pBR322 DNA and relaxed pGEM3 DNA, the electrophoresis being done without chloroquine in the first dimension and with 4 Ìg/ml chloroquine in the second dimension. Lanes 1-3 involved initial incubation at 300 mM NaCl with one of the two DNAs, followed by adjustment of NaCl concentration to 50 mM and addition of the competitor DNA. After raising the temperature to 90°C, incubation was for 15 minutes. In lanes 6-8, NaCl concentration remained at 300 mM. In lanes 1 and 6, both 0.2 Ìg relaxed pBR322 DNA and 0.2 Ìg relaxed pGEM3 DNA were present at the start of incubation.

In lanes 2 and 7, 0.2 ìg relaxed pBR322 DNA was present at the start of incubation, and 0.2 ìg pGEM3 DNA was added after dilution or reduction of NaCl concentration. In lanes 3 and 8, 0.2 ìg relaxed pGEM3 DNA was present at the start of incubation, and 0.2 ìg pBR322 DNA was added after dilution or reduction of NaCl concentration. Lane 4 contained relaxed pGEM3, lane 5 relaxed pBR322, lane 9 negatively supercoiled pGEM3, and lane 10, negatively supercoiled pBR322 DNA.

Figure 14B is similar to Figure 14A except that the composite preparation of pGEM3 contained supercoiled and relaxed pGEM3 was used, and incubation at 90°C was for 10 minutes.

Irrespective of whether the substrate was pBR322 DNA and the competitor was pGEM3 or vice-versa, whether both substrate and competitor were relaxed, the substrate was relaxed and the competitor was supercoiled, or vice-versa, in all cases at 300 mM NaCl the enzyme unlinked both substrate and competitor DNA (Figs. 14A, 14B). This means that at this salt concentration topoisomerase freely disassociates and binds DNA. However, if the NaCl concentration was adjusted to 50 mM before competitor DNA was added, the degree of reaction on the competitor DNA was very low.

Example 9
Inhibitors of Topoisomerase V

Camptothecin is not as potent an inhibitor of *M. kandleri* topoisomerase V as of many eukaryotic topoisomerase I species is. Camptothecin is soluble in dimethyl sulfoxide, an organic solvent that does not inhibit eukaryotic topoisomerases. However, dimethyl sulfoxide alone, without camptothecin, inhibits *M. kandleri* topoisomerase V (Fig. 15).

Figure 15 is a photograph of an agarose gel showing the effect of dimethyl sulfoxide and camptothecin on the activity of topoisomerase V (15 units) with 0.2 Ìg pBR322 DNA as substrate at 70°C. The indicated concentrations of dimethyl sulfoxide (DMSO) and camptothecin (CPT) were used. Incubations were carried out for 15 minutes.

Example 10

Covalent Complex Formation Between Topoisomerase V and DNA

M. kandleri topoisomerase V forms a covalent complex with DNA. This is demonstrated in Figure 16. Uniformly labeled pBR322 DNA was prepared with the T7 QuickPrime kit (Pharmacia) and [Á-^{32}P]dCTP (NEN). Labeled DNA (5 ng) was incubated with 40 ng of topoisomerase V in 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl at 70°C for three minutes. Then KOH was added to 0.05 N and incubation was continued for one minute. The mixture was neutralized by Tris-HCl and magnesium acetate was added to 10 mM. The DNA was digested by 20 units of DNase I and 300 units of exonuclease III (Pharmacia) at 37°C for 50 minutes. The products were analyzed by SDS-PAGE on a 4-15% gradient gel. The silver-stained gel (lane 1) and its autoradiograph (lane 2) are shown (Fig. 16). These results show that DNA forms a covalent complex with topoisomerase V.

Example 11

DNA Cleavage by *M. kandleri* Topoisomerase V

As indicated above (Example 5; Fig. 11), topoisomerase V is a type 1 topoisomerase that makes cuts in one strand at a time. The exact site of cleavage was determined as shown in Figure 17. pGEM1 DNA (Promega) was cut with *Hind* III restriction endonuclease and the 3'-end was filled in with [Á-^{32}P]dCTP and unlabeled

deoxyribonucleoside triphosphates by the Klenow fragment of Escherichia coli DNA polymerase I, followed by digestion with Nae I restriction endonuclease. The 280-bp end-labeled fragment was gel purified and incubated with (Fig. 17, lane 2) or without (Fig. 17, lane 1) topoisomerase V. The samples were boiled in running buffer (6 M urea, 0.5 x TBE) and analyzed using denaturing 6% polyacrylamide gel electrophoresis. The major cleavage site is shown by an arrow.

The exact position of the major topoisomerase V cleavage site on the 280-bp fragment of pGEM 1 DNA was determined by comparing the mobility of the cleavage product with the Maxam-Gilbert sequencing ladder. The sequence is shown in Figure 18. The sequence is similar, though not identical, to the strongest cleavage sites for eukaryotic topoisomerases I (B.J. Bonven et al. Cell 41: 541-551 (1985)).

Example 12

Isolation of Covalent Complex Between M. kandleri Topoisomerase V and Open Circular DNA

Covalent complexes between M. kandleri topoisomerase V enzyme were isolated for three different plasmids: pHOT1 containing the consensus cleavage site for eukaryotic topoisomerase I inserted in the pUC12 vector, pGEM3, and pBR322. Relaxed DNA (0.2 ìg for each plasmid) was incubated in 30 mM Tris-HCl, pH 8.0 at 25°C, 300 mM NaCl, 5 mM magnesium acetate with or without topoisomerase V (100 units) at 90°C for 3 minutes. Sodium dodecyl sulfate was added to 1% and the resulting mixtures were incubated for 90°C for 1 minute. A similar reaction was performed using supercoiled DNA for each plasmid and human topoisomerase I with NaCl at 85 mM. For each assay condition (i.e., each plasmid, relaxed or supercoiled, with or without M. kandleri topoisomerase V or human

topoisomerase I), an aliquot was digested with 2 ìg proteinase K at 42°C for 4 hours.

Two-dimensional 1.5% agarose gel electrophoresis was performed in TAE buffer with 0.1% SDS in the first dimension and with 4 ìg/ml chloroquine in the second dimension.

The results are shown in Fig. 19 for each of the three plasmids (pHOT1, top; pGEM3, center; pBR322 (bottom)). For each plasmid, the lanes shown are as follows: lane 1, untreated supercoiled DNA; lane 2, relaxed DNA incubated with *M. kandleri* topoisomerase V; lane 3, relaxed DNA incubated with *M. kandleri* topoisomerase V and digested with proteinase K; lane 4, relaxed DNA without *M. kandleri* topoisomerase V; lane 5, relaxed DNA without *M. kandleri* topoisomerase V and digested with proteinase K; lane 6, untreated relaxed DNA; lane 7, supercoiled DNA incubated with human topoisomerase I; lane 8, supercoiled DNA incubated with human topoisomerase I and digested with proteinase K; lane 9, supercoiled DNA without human topoisomerase I; and lane 10, supercoiled DNA without human topoisomerase I and digested with proteinase K. Lane 1 also contained 1 ìg pGEM3 DNA.

Lane 2 for all three plasmids tested shows that the incubation converts relaxed DNA to a highly unwound form. In addition, a band (indicated by an arrow) above open circular DNA appears. Lane 3 shows that this product is sensitive to proteinase K, which indicates that it is a covalent complex of *M. kandleri* topoisomerase V with circular DNA fixed by the protein denaturant. Proteinase K digests topoisomerase and yields a nicked circle of DNA with one or a few amino acids attached to the DNA, which co-migrates with open circular DNA.

Only about 1% of the enzyme molecules were trapped. There was no substantial difference among the three substrate DNAs used in the yield of the complexes, even though pHOT1 has the consensus site for topoisomerase I and pGEM3 and pBR322 lack the site.

For comparison, covalent complex formation by human topoisomerase V is also shown (lanes 7 and 8). The results are similar to those obtained with M. kandleri topoisomerase V.

Example 13 Proteolytic Fragments of the M. kandleri Topoisomerase V

Treatment of the M. kandleri topoisomerase V with endoproteinase Lys-C, a protease that cleaves at the carboxylic side of lysine (K), generates two large fragments which are relatively stable to further digestion (Fig. 20)

Samples of topoisomerase V (10 ìg/ml and 35 ìg/ml) were incubated with 0.1 ìg/ml Lys-C (Promega, Madison, WI) in 30 mM sodium phosphate, pH 8.0, 0.5 M NaCl, 10 mM ß-mercaptoethanol for 30 and 150 min at 37°C. Reactions were terminated by adding 1 mM TLCK. Fractions were analyzed by SDS-PAGE (7.5%) and stained by silver.

For large-scale proteolysis, about 100 ìg of topoisomerase V was digested with 3 ìg of Lys-C in the above buffer for 3 h. Fragments were separated by 6% SDS-PAGE and blotted onto Immobilon P membrane (Millipore, Bedford, MA).

Figure 20 shows that molecular masses of the fragments are about 75 kD and 33 kD. Determination of the N-terminal sequences of these fragments showed that they are derived from the N terminus (75-kD fragment) and from the C terminus (33-kD fragment) of topoisomerase V. The

33-kD fragment, designated as Fragment IV, has the following N-terminal amino acid sequence: K-S-G-R-Q-E-R-S-E-E-E-E-K-E-E-L-E-R-K-V-G-E-G-R-A-R-R-L-I-E-Y-F-G-S-A.

The fragments were found to be resistant to further digestion under the conditions of Figure 20 and could represents structural and/or functional domains within M. kandleri topoisomerase V . However, prolonged incubation and increased Lys-C:topoisomerase V ratio leads to appearance of a number of additional fragments (Fig. 21), including a N-terminal fragment of about 55,000 daltons.

ADVANTAGES OF THE INVENTION

Thermostable topoisomerase V from Methanopyrus kandleri can function over a broad range of temperatures and ionic conditions to promote both relaxation of supercoils and unlinking of ccDNA. The enzyme has many applications in enzymatic DNA sequencing, in gene cloning, in nucleic acid amplification by the PCR or LCR techniques, and in hybridization probe preparation, *i.e.*, in any procedure that requires denaturing of ccDNA or annealing of primers or hybridization probes.

Although the present invention has been described in considerable detail with regard to certain preferred versions thereof, other versions are possible. Therefore, the spirit and scope of the appended claims should not be limited to the description of the preferred version contained herein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Slesarev, Alexei I

(ii) TITLE OF INVENTION: Thermostable Prokaryotic DNA Topoisomerase V

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Los Angeles
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(F) ZIP: 90025

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US94/03217
(B) FILING DATE: 24-MAR-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Farber, Michael B
(B) REGISTRATION NUMBER: 32,612
(C) REFERENCE/DOCKET NUMBER: 30444.1-WO-01

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (310) 445-1140
(B) TELEFAX: (310) 445-9031

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Methanopyrus kandleri

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Ala Leu Val Tyr Asp Ala Glu Phe Val Gly Ser Glu Arg
1               5                   10
Glu Phe Glu Glu Glu Arg Glu Thr Phe Leu Lys Gly
    15                  20                  25

Val Lys Ala Tyr Asp Gly Val Leu Ala Thr Ile Pro Phe Leu
                30                  35
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Methanopyrus kandleri

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Lys Ser Asp Thr Glu Thr Ile Glu Thr
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Methanopyrus kandleri

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Lys Pro Glu Leu Pro Tyr Val Ala Val Pro Pro His Met
1 5 10

Ala Glu Arg Ala Arg Arg Val Leu Thr Arg Glu Asp Asp
15 20 25

Leu Ala Xaa Asp Val Xaa Ala
30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Methanopyrus kandleri

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Arg Val Pro Arg Ala Xaa Xaa Gly Xaa Xaa Phe Asp
1 5 10

Arg Leu
15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Methanopyrus kandleri

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Lys Ser Gly Arg Gln Glu Arg Ser Glu Glu Glu Glu Lys
1 5 10

Glu Glu Leu Glu Arg Lys Val Gly Glu Gly Arg Ala
15 20 25

Arg Arg Leu Ile Glu Tyr Phe Gly Ser Ala
30 35

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Methanopyrus kandleri

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Lys Tyr Gly Ser Ala Ser Xaa Xaa Arg Arg Leu Pro Xaa
1               5                   10

Glu Glu Xaa Arg Glu Leu Gly Phe Xaa Asp Asp Arg
    15                  20                  25
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: pBR322 plasmid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACACTATAGA ATACAAG 17

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Consensus sequence for topoisomerase I

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGACTTNAGA RAAAWWW 17

What is claimed is:

1. Substantially purified thermostable DNA topoisomerase from Methanopyrus kandleri, the topoisomerase being substantially free of other enzymes acting on DNA, the topoisomerase being a Type 1-Group B topoisomerase whose reaction precedes via a transient single-strand break and changes the linking number in steps of 1 and operates on duplex DNA, binding covalently to the 3'-phosphoryl terminus of the DNA cleaved.

2. The DNA topoisomerase V of claim 1 that is a single chain polypeptide with an estimated molecular weight of about 110,000 daltons, based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

3. A process for preparing substantially purified thermostable DNA topoisomerase V from Methanopyrus Kandleri, comprising the steps of:

(a) lysing cells of Methanopyrus Kandleri to form a lysate;

(b) treating the lysate with polyethyleneimine to form a precipitate and a supernatant;

(c) precipitating the polyethyleneimine supernatant with ammonium sulfate to form an ammonium sulfate precipitate;

(d) chromatographing the ammonium sulfate precipitate on phosphocellulose to produce a phosphocellulose eluate;

(e) chromatographing the phosphocellulose eluate on heparin to produce a heparin eluate;

(f) chromatographing the heparin eluate on a column to separate proteins by molecular size therein to produce a substantially purified thermostable DNA topoisomerase V; and

(g) recovering the substantially purified thermostable topoisomerase.

4. The product produced by the process of claim 3.

5. A method of relaxing supercoiled DNA comprising treating a supercoiled DNA selected from the group consisting of positively supercoiled DNA and negatively supercoiled DNA with the DNA topoisomerase V of claim 1 at a temperature and ionic conditions at which the separation of complementary DNA strands of the linear form of treated closed circular DNA does not occur and at a temperature and ionic conditions that allow the enzyme to bind to DNA and catalyze the relaxation reaction to produce at least partially treated DNA.

6. A method of unlinking closed circular DNA comprising treating closed circular DNA with the topoisomerase V of claim 1 at a temperature and ionic conditions allowing partial or complete separation of complementary DNA strands of the linear form of treated closed circular DNA and at a temperature and ionic conditions that allow the enzyme to bind to DNA and catalyze the unlinking reaction to produce DNA with a linking number lower than the linking number of the DNA before treatment.

7. A complex comprising the topoisomerase of claim 1 non-covalently bound to DNA.

8. A thermostable Type 1-Group B DNA topoisomerase possessing an amino-terminal amino acid sequence selected from the group consisting of:

(a) the amino-terminal amino acid sequence of Methanopyrus kandleri topoisomerase V as follows: A-L-V-Y-D-A-E-F-V-G-S-E-R-E-F-E-E-E-R-E-T-F-L-K-G-V-K-A-Y-D-G-V-L-A-T-I-P-F-L; and

(b) an amino-terminal amino acid sequence related to the amino-terminal amino acid sequence of Methanopyrus kandleri topoisomerase V by one or more conservative amino acid substitutions; the topoisomerase being magnesium independent, relaxing both negatively and positively supercoiled DNA, making a covalent complex with the 3'-end of the broken DNA strand, and cleaving DNA at the third adenosine residue in the sequence A-C-A-C-T-A-T-A-G-A-A-T-A-C-A-A-G.

9. The type 1-group B DNA topoisomerase of claim 8 wherein the amino-terminal amino acid sequence is the amino-terminal amino acid sequence of M. kandleri topoisomerase V.

10. A thermostable type 1-Group B DNA topoisomerase isolated from a thermophilic prokaryote possessing at least one amino acid sequence selected from the group consisting of:

(a) the sequence K-S-D-T-E-T-I-E-T;

(b) the sequence K-P-E-L-P-Y-V-A-V-P-P-H-M-A-E-R-A-R-R-V-L-T-R-E-D-D-L-A-X-D-V-X-A;

(c) the sequence K-R-V-P-R-A-X-X-G-X-X-F-D-R-L;

(d) the sequence K-S-G-R-Q-E-R-S-E-E-E-E-K-E-E-L-E-R-K-V-G-E-G-R-A-R-R-L-I-E-Y-F-G-S-A;

(e) the sequence K-Y-G-S-A-S-X-X-R-R-L-P-X-E-E-X-R-E-L-G-F-X-D-D-R in which X represents an unidentified amino acid; and

(f) a sequence related to the sequences recited in (a), (b), (c), (d) or (e) by one or more conservative amino acid substitutions; the topoisomerase being magnesium independent, relaxing both negatively and positively supercoiled DNA, making a covalent complex with the 3'-end of the broken DNA strand, and cleaving DNA at the third adenosine residue in the sequence A-C-A-C-T-A-T-A-G-A-A-T-A-C-A-A-G.

11. The type 1-group B topoisomerase of claim 10 wherein the at least one amino acid sequence is selected from the group consisting of the sequence K-S-D-T-E-T-I-E-T, the sequence K-P-E-L-P-Y-V-A-V-P-P-H-M-A-E-R-A-R-R-V-L-T-R-E-D-D-L-A-X-D-V-X-A, the sequence K-R-V-P-R-A-X-X-G-X-X-F-D-R-L, the sequence K-S-G-R-Q-E-R-S-E-E-E-E-K-E-E-L-E-R-K-V-G-E-G-R-A-R-R-L-I-E-Y-F-G-S-A, and the sequence K-Y-G-S-A-S-X-X-R-R-L-P-X-E-E-X-R-E-L-G-F-X-D-D-R in which X represents an unidentified amino acid.

12. Antibody specifically binding the substantially purified thermostable DNA topoisomerase of claim 1.

13. The antibody of claim 12 that is a monoclonal antibody.

14. Antibody specifically binding a fragment of about 75,000 daltons of the substantially purified topoisomerase of claim 1 originating from the amino-terminus of the topoisomerase protein.

15. Antibody specifically binding a fragment of about 33,000 daltons of the substantially purified topoisomerase of claim 1 originating from the carboxyl-terminus of the topoisomerase protein.

16. A method of detecting and/or determining the M. kandleri topoisomerase V of claim 1 comprising the steps of:

(a) reacting the topoisomerase of claim 1 with an anti-human topoisomerase I antibody; and

(b) detecting and/or determining the topoisomerase V by detecting and/or determining an antigen-antibody complex between the topoisomerase and the antibody.

17. A method of relaxing supercoiled DNA comprising treating a supercoiled DNA selected from the group consisting of positively supercoiled DNA and negatively supercoiled DNA with the prokaryotic type 1, Group B topoisomerase of claim 8 at a temperature and ionic conditions at which the separation of complementary strands of the linear form of treated closed circular DNA does not occur and at a temperature and ionic conditions that allow the enzyme to bind to DNA and catalyze the relaxation reaction to produce at least partially treated DNA.

18. A method of unlinking closed circular DNA comprising treating closed circular DNA with the prokaryotic type 1, Group B topoisomerase of claim 8 at a temperature and ionic conditions allowing partial or complete separation of complementary DNA strands of the linear form of treated closed circular DNA and at a temperature and ionic conditions that allow the enzyme to bind to DNA and catalyze the unlinking reaction to produce DNA with a linking number lower than the linking number of the DNA before treatment.

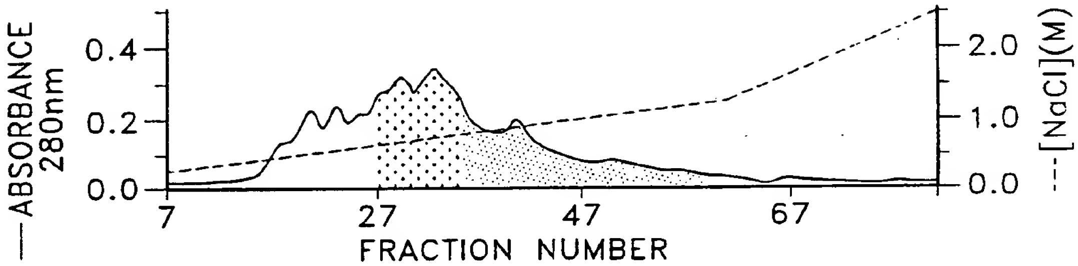


FIG. 1A

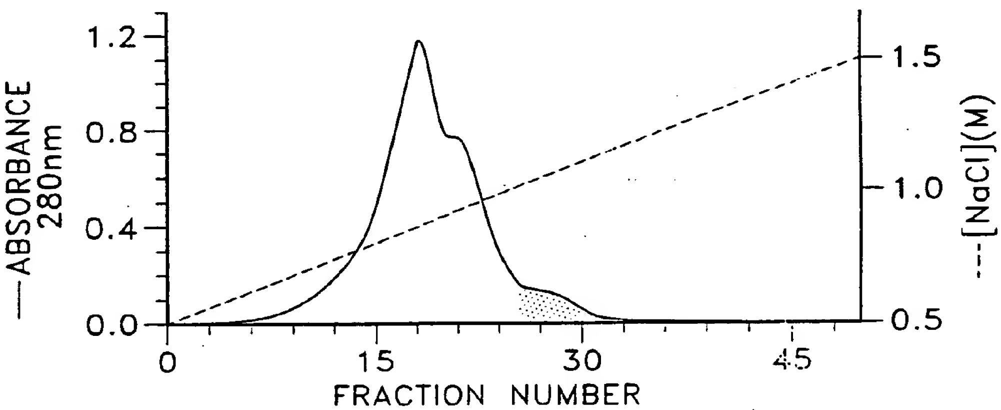


FIG. 2A

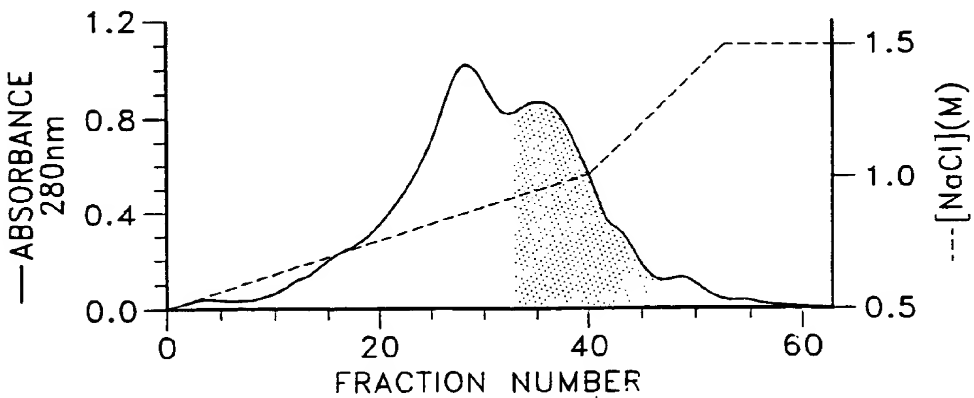


FIG. 3A

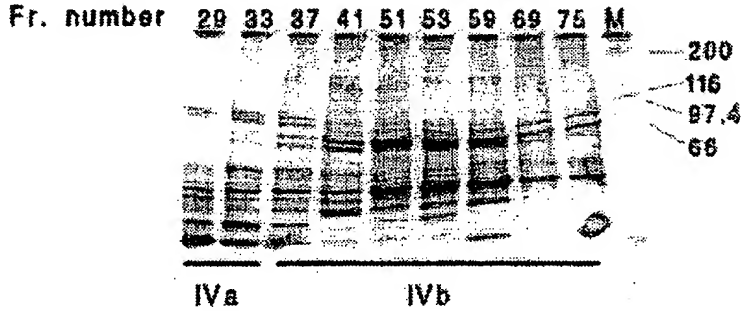


FIG. 1B

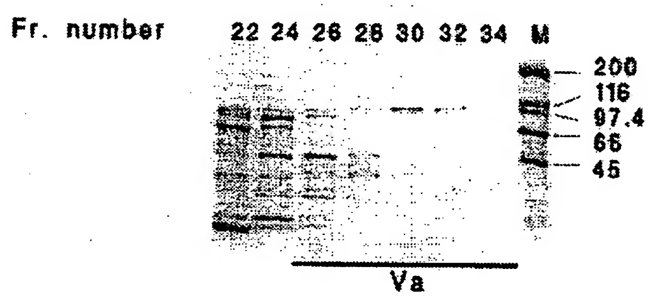


FIG. 2B

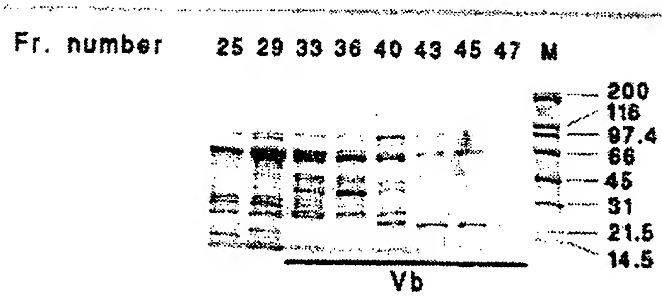


FIG. 3B

FIG. 4A

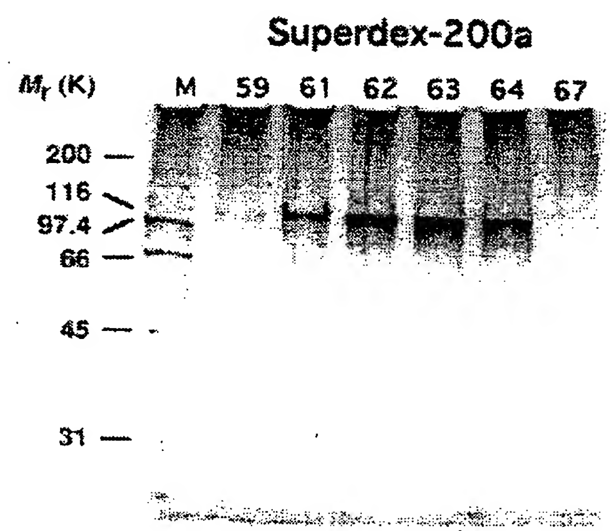
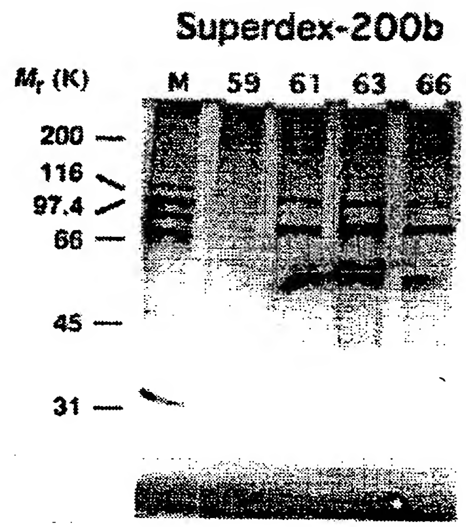


FIG. 4B



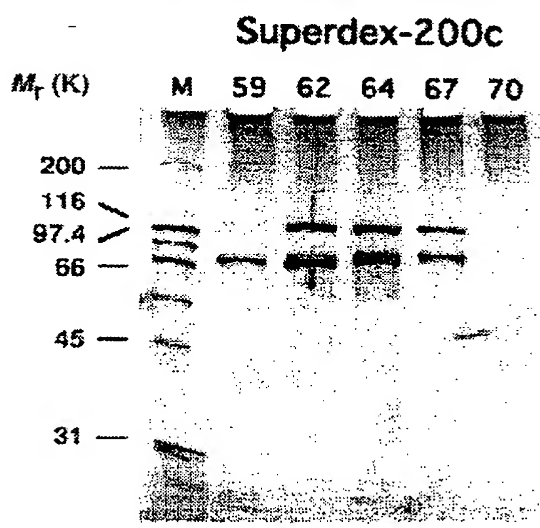


FIG. 4C

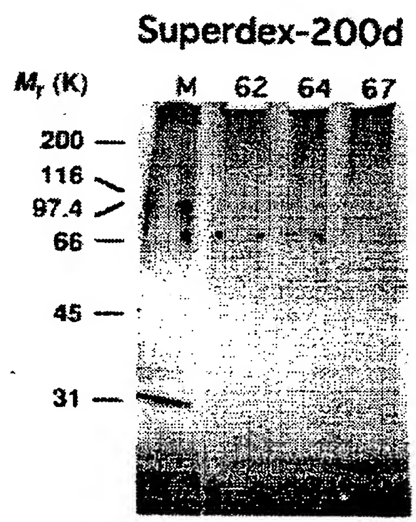


FIG. 4D

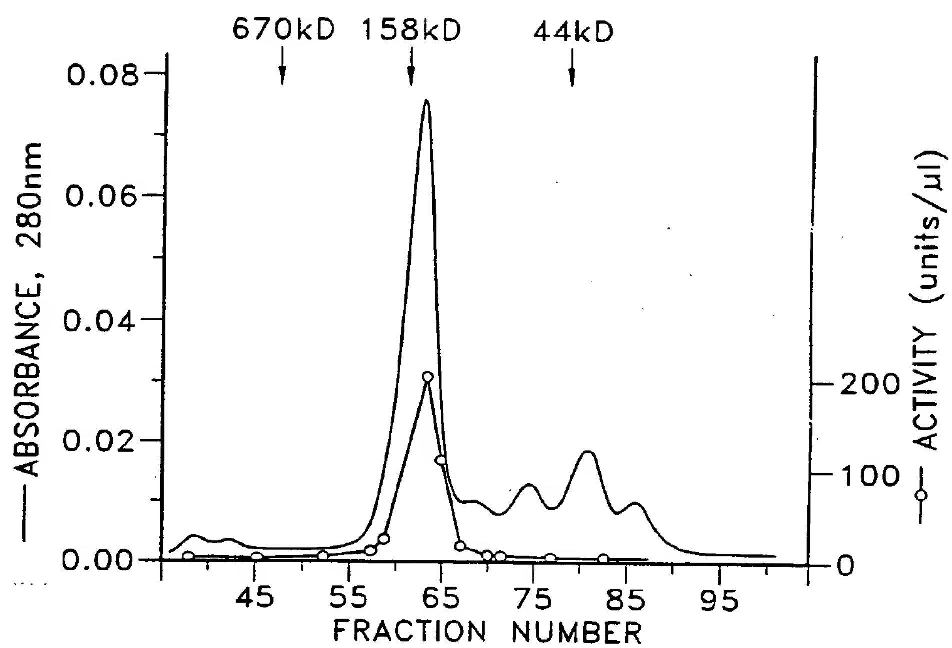


FIG. 5A

```
                                   ↓
STRONG TOPO V SITE     ACACTA  TAGAATACAAG
                       *  ***  ****  *  **
CONSENSUS TOPO I SITE  AGACTT -AGAAAAAAAA
                                  G    TTT
```

FIG. 18

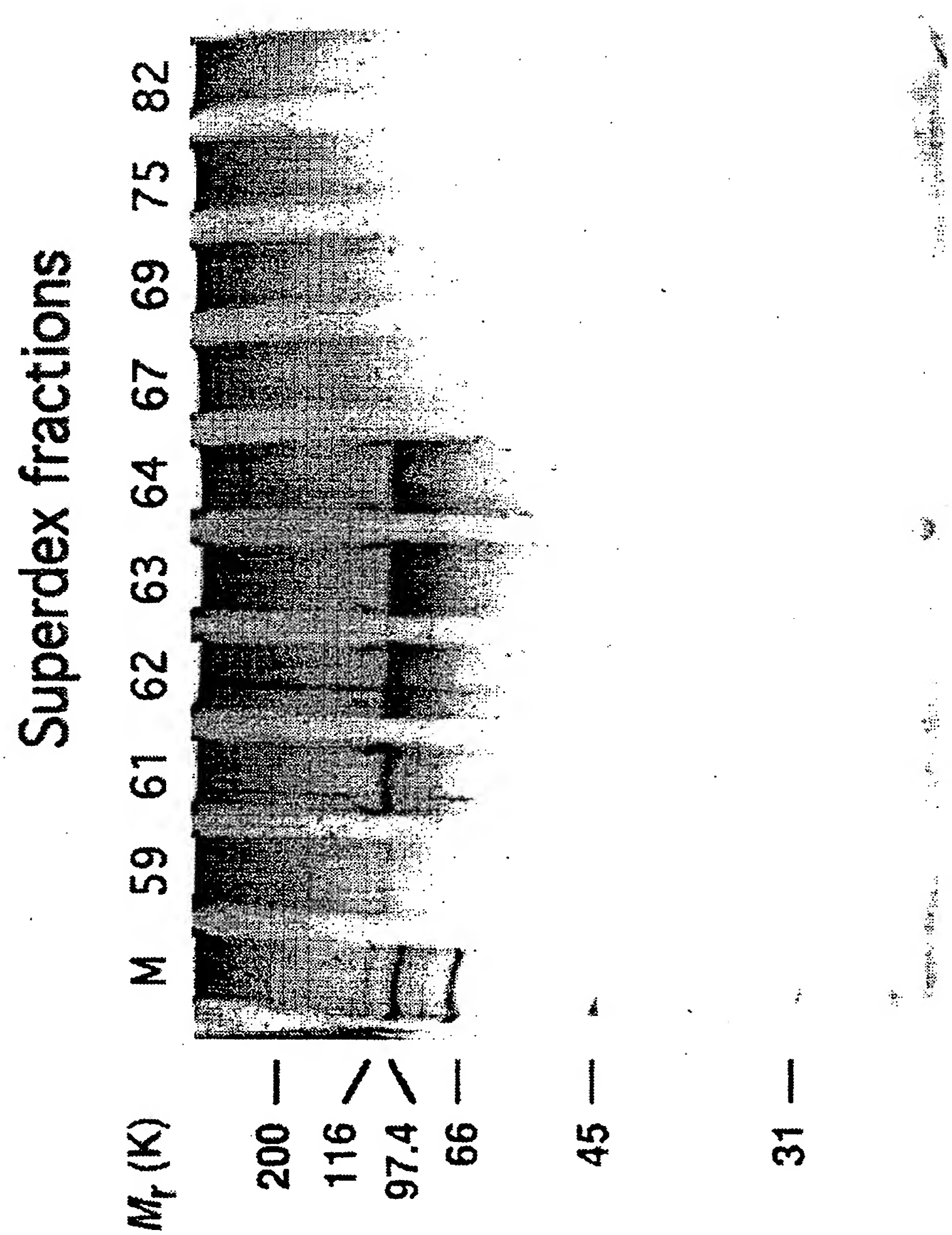

Superdex fractions
M 59 61 62 63 64 67 69 75 82
M_r (K)
200
116
97.4
66
45
31

FIG. 5B

Western blot with anti-human topo I antibodies

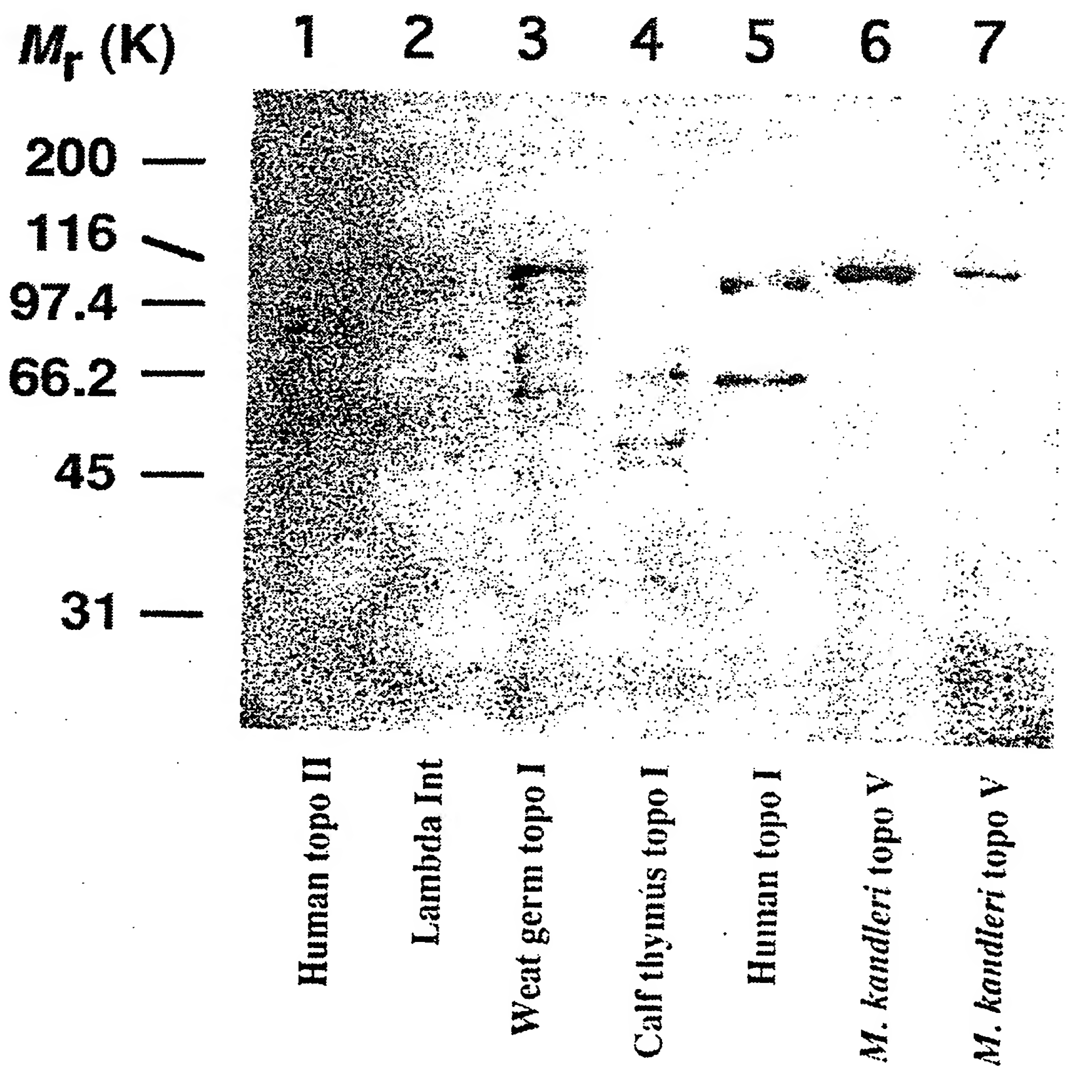


FIG. 6

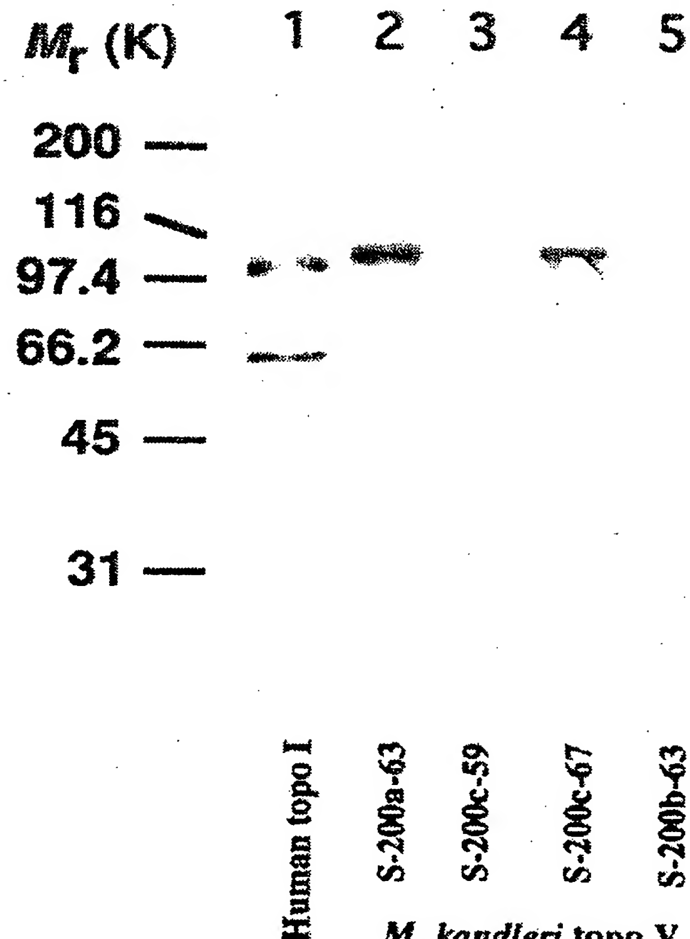

M_r (K)
1 2 3 4 5
200
116
97.4
66.2
45
31
Human topo I
S-200a-63
S-200c-59
S-200c-67
S-200b-63
M. kandleri topo V

FIG. 7

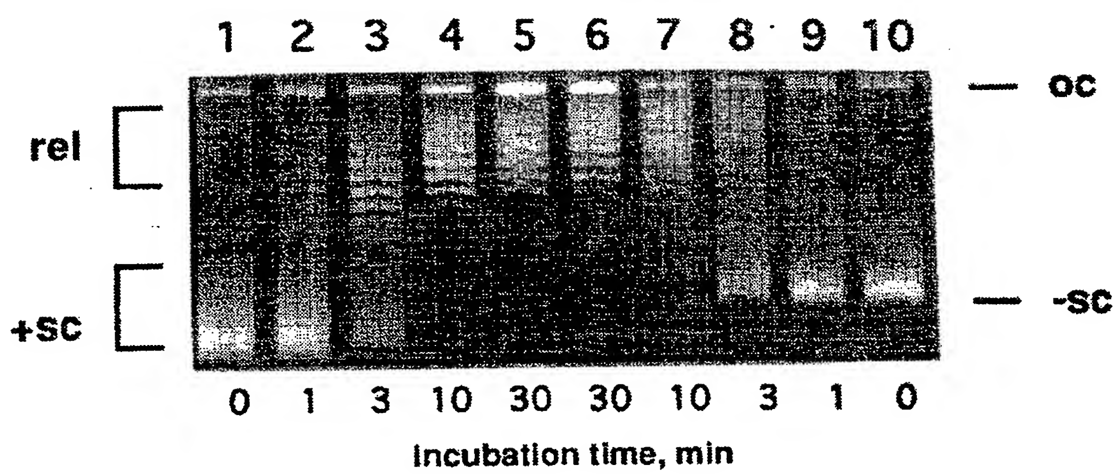

1 2 3 4 5 6 7 8 9 10
oc
rel
+SC
-SC
0 1 3 10 30 30 10 3 1 0
Incubation time, min

FIG. 8A

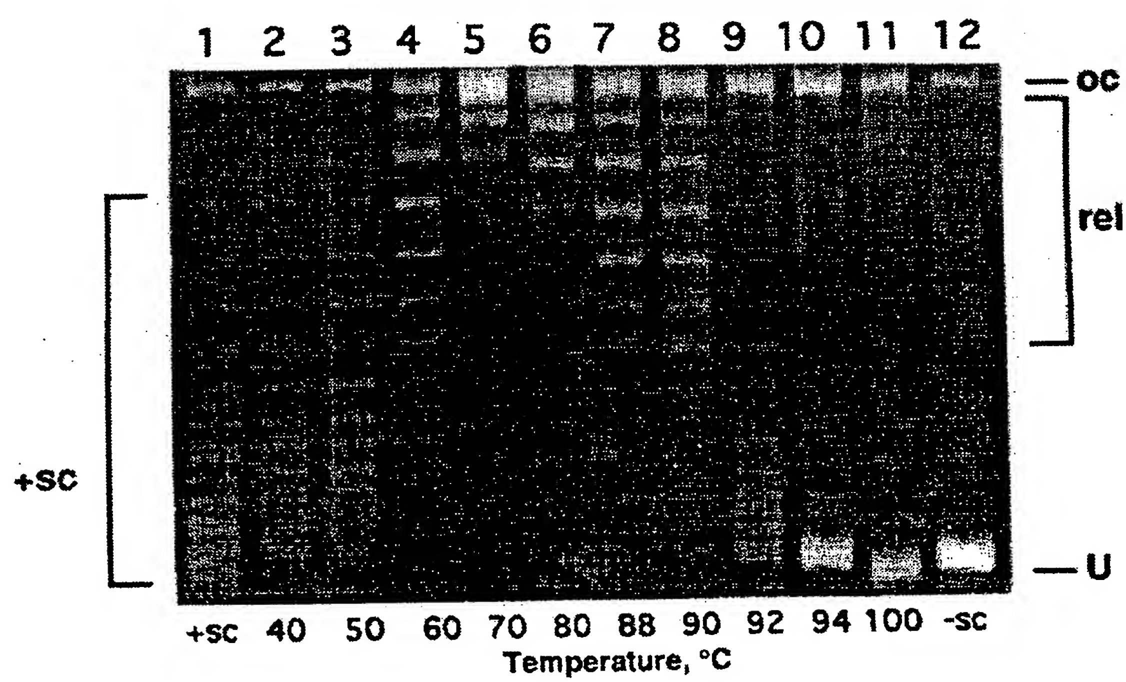

1 2 3 4 5 6 7 8 9 10 11 12
oc
rel
+SC
U
+sc 40 50 60 70 80 88 90 92 94 100 -sc
Temperature, °C

FIG. 8B

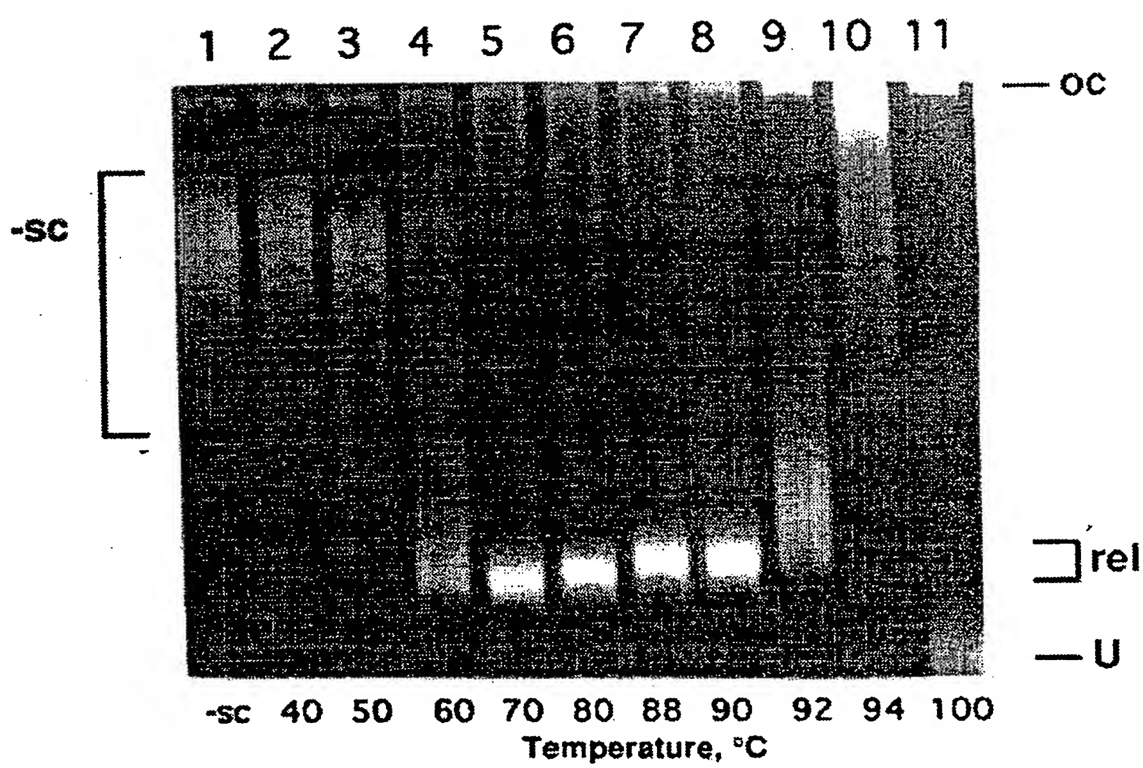
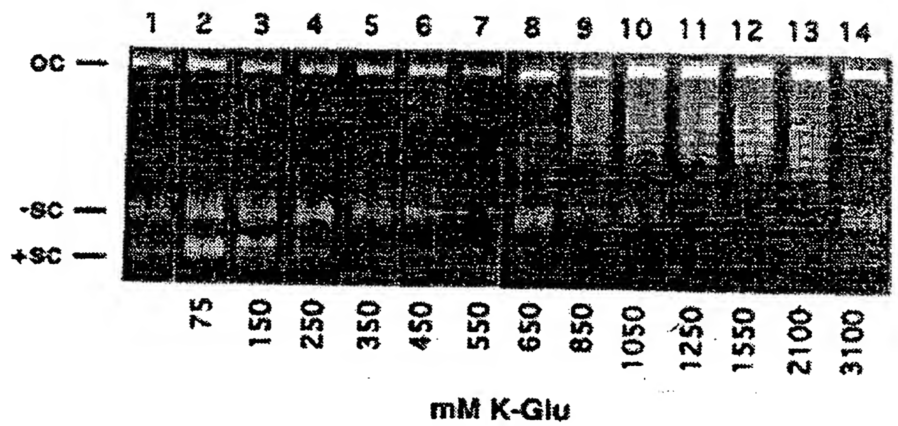
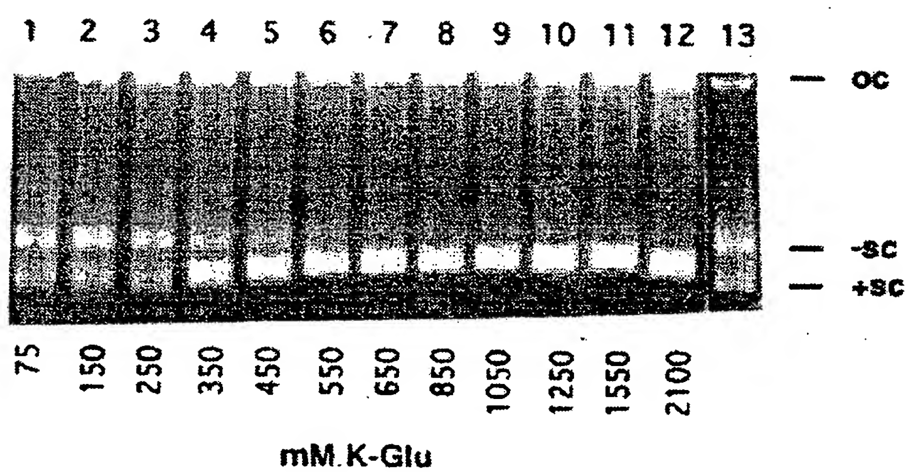

1 2 3 4 5 6 7 8 9 10 11
oc
-sc
rel
U
-sc 40 50 60 70 80 88 90 92 94 100
Temperature, °C

FIG. 8C



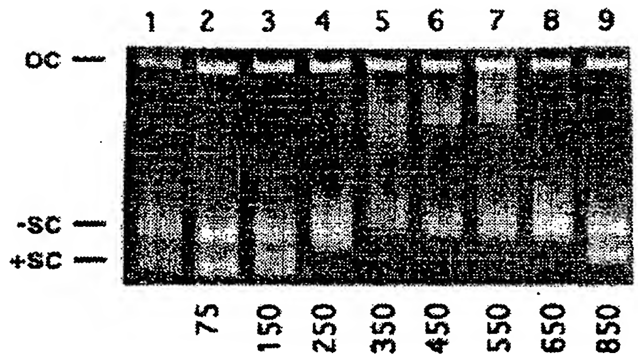
5 mM Na_2EDTA, 88°C

FIG. 9A



5 mM Na_2EDTA, 95°C

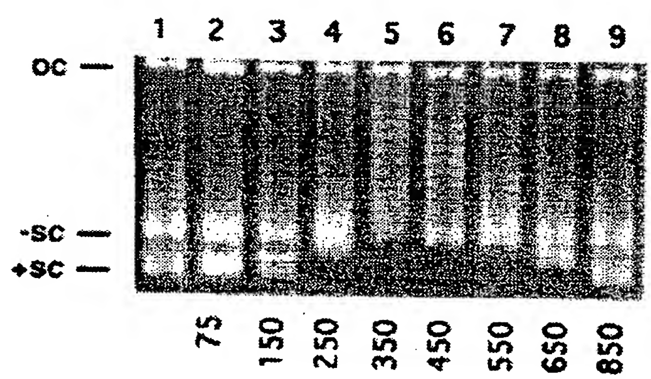
FIG. 9D



mM KCl

5 mM Na_2EDTA, 88°C

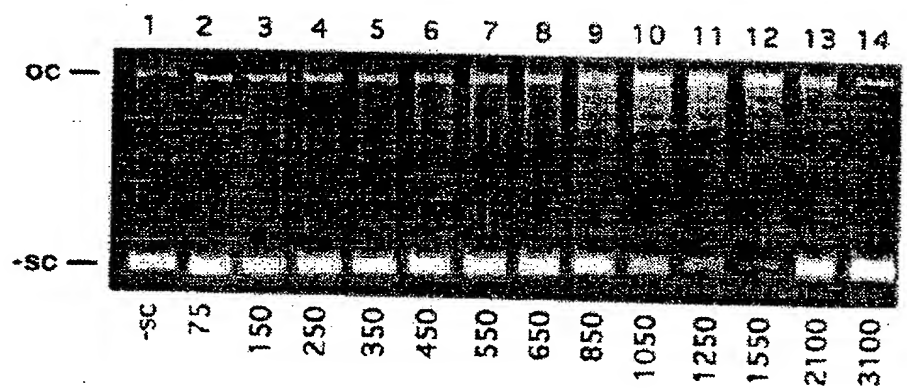
FIG. 9B



mM NaCl

5 mM Na_2EDTA, 88°C

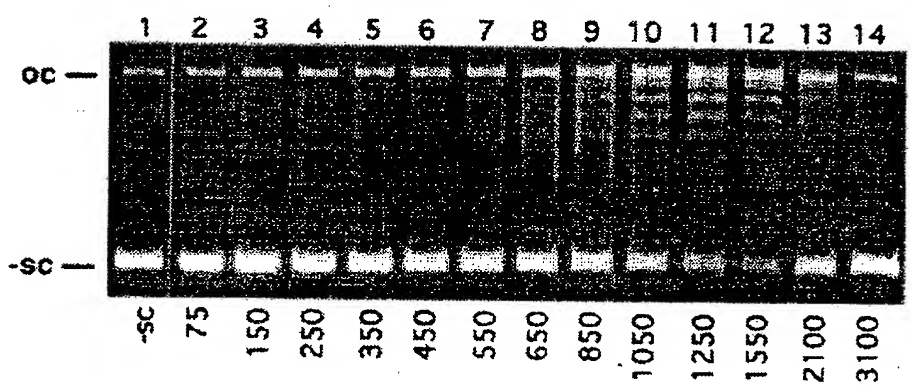
FIG. 9C



mM K-Glu

5 mM Na_2EDTA, 80°C

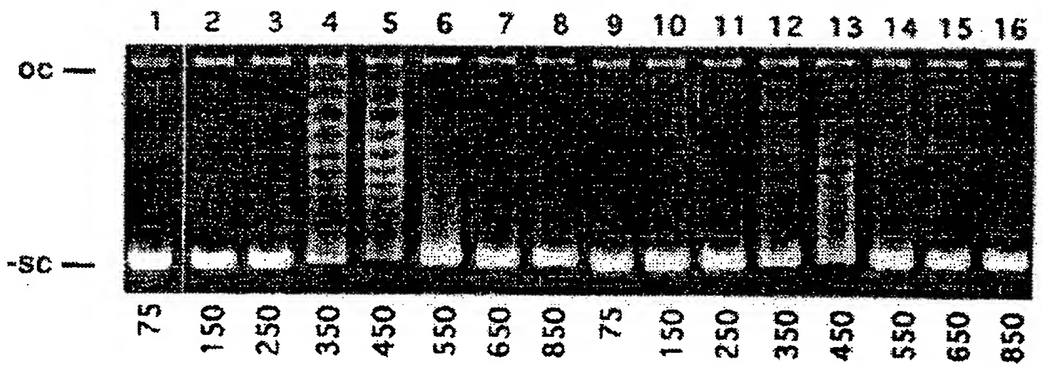
FIG. 10A



mM K-Glu

5 mM $MgCl_2$, 80°C

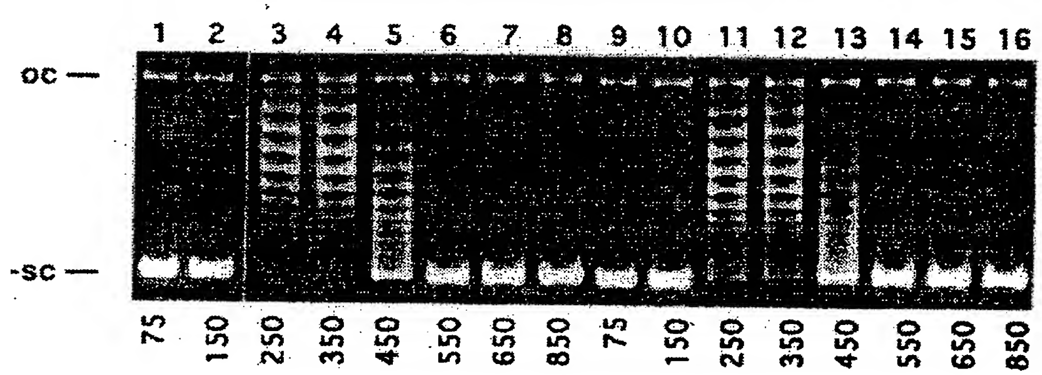
FIG. 10B



mM KCl, 80°C

5 mM Na_2EDTA 5 mM $MgCl_2$

FIG. 10C



mM NaCl, 80°C

5 mM Na_2EDTA 5 mM $MgCl_2$

FIG. 10D

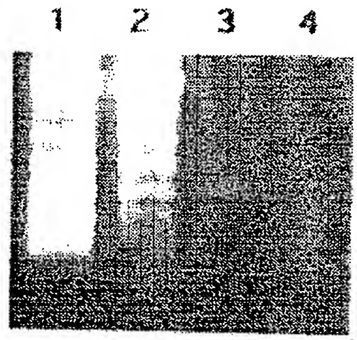

1 2 3 4

FIG. 11

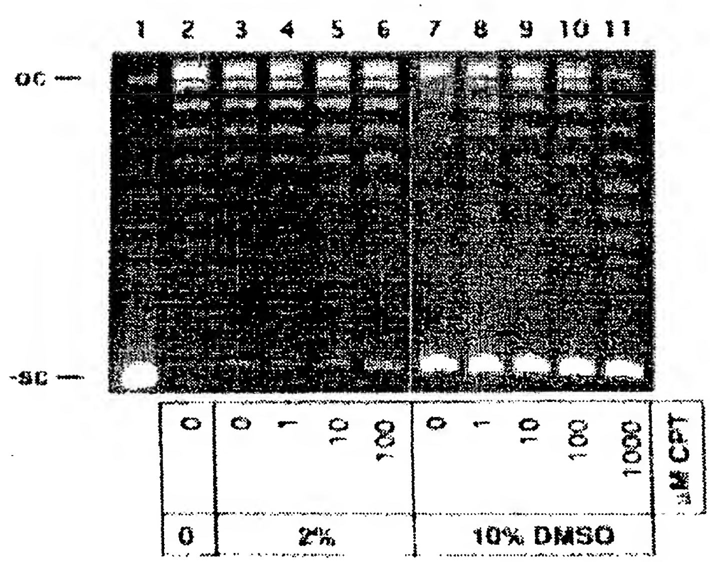

1 2 3 4 5 6 7 8 9 10 11
OC
SC
0 0 1 10 100 0 1 10 100 1000
μM CPT
0
2%
10% DMSO

FIG. 15

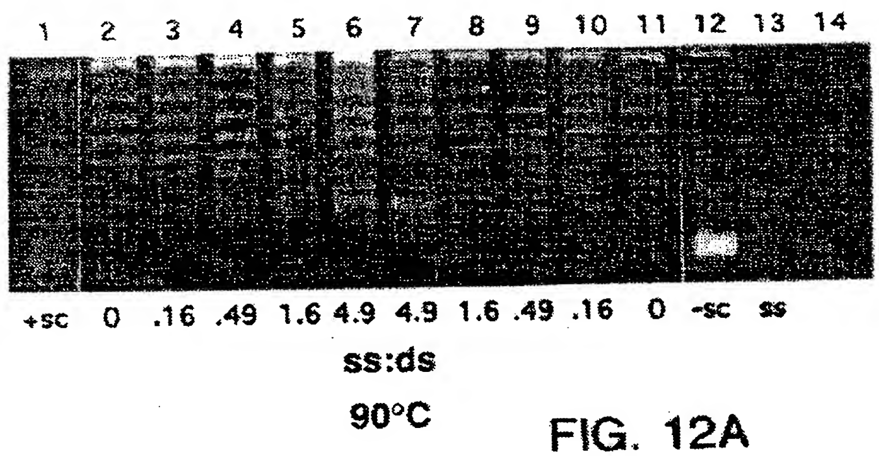


FIG. 12A

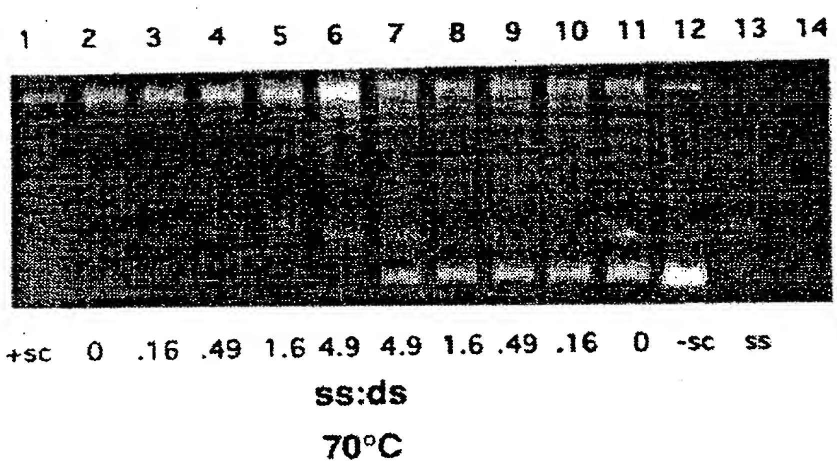


FIG. 12B

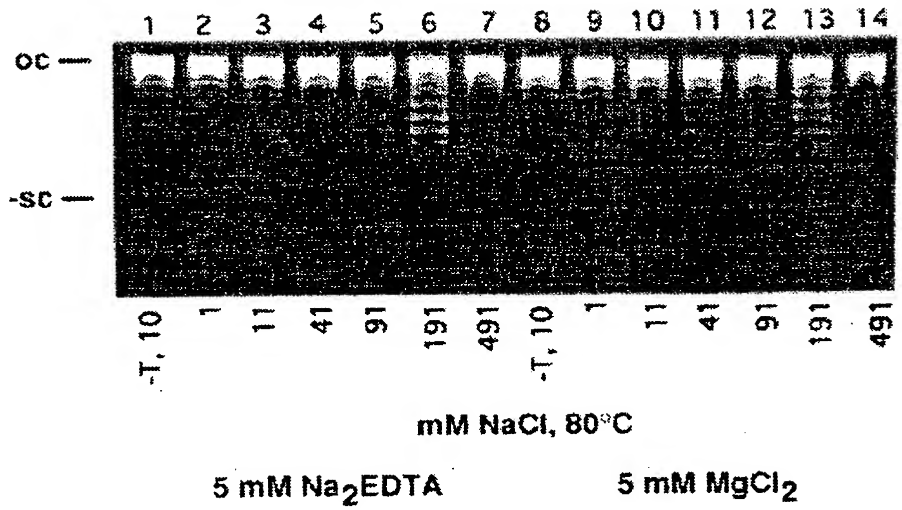


FIG. 13A

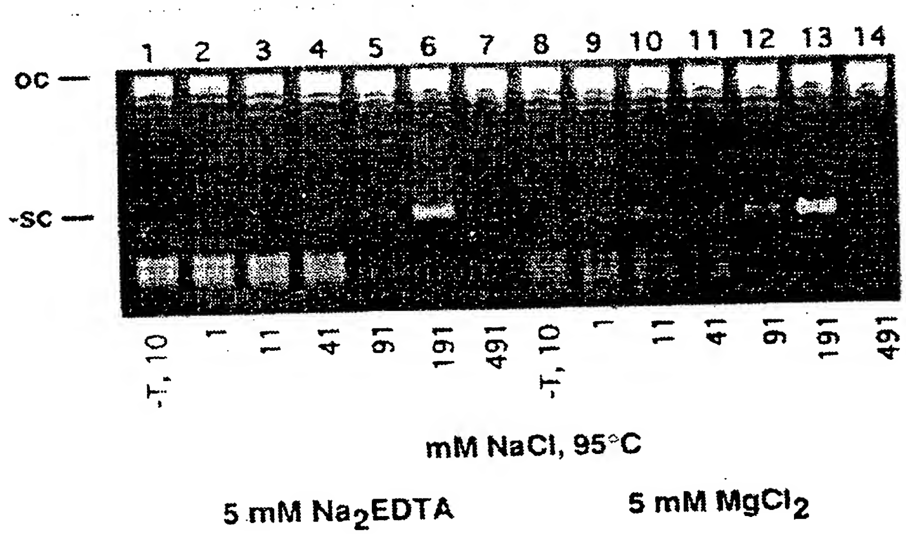


FIG. 13B

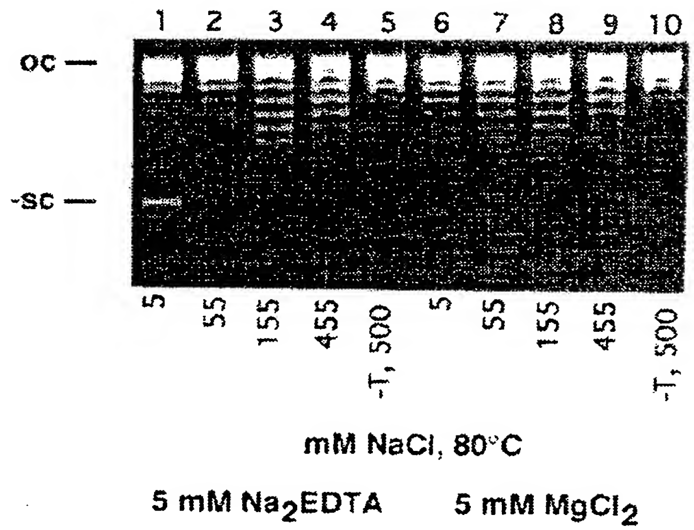


FIG. 13C

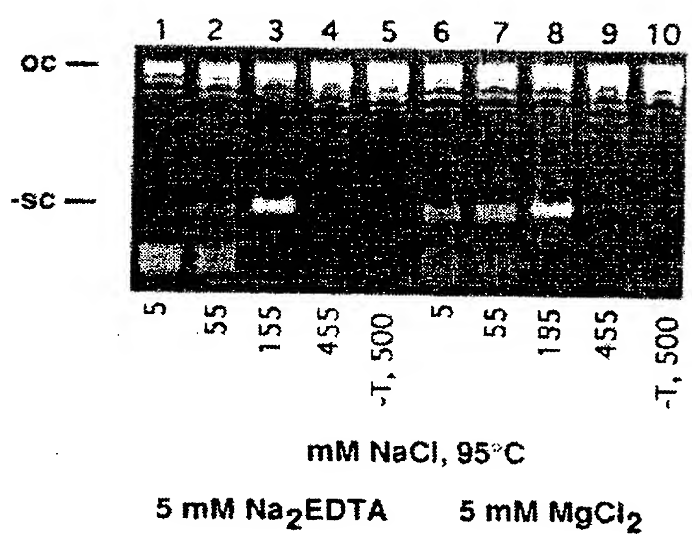


FIG. 13D

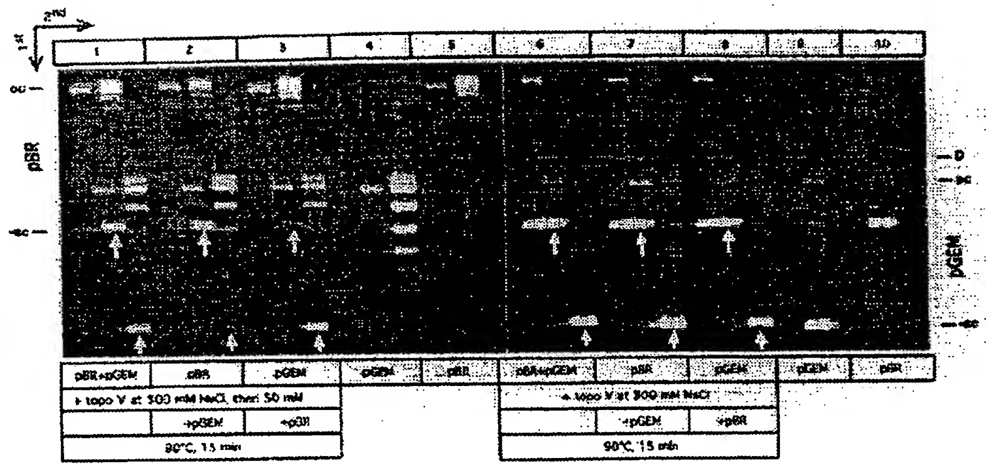


FIG. 14A

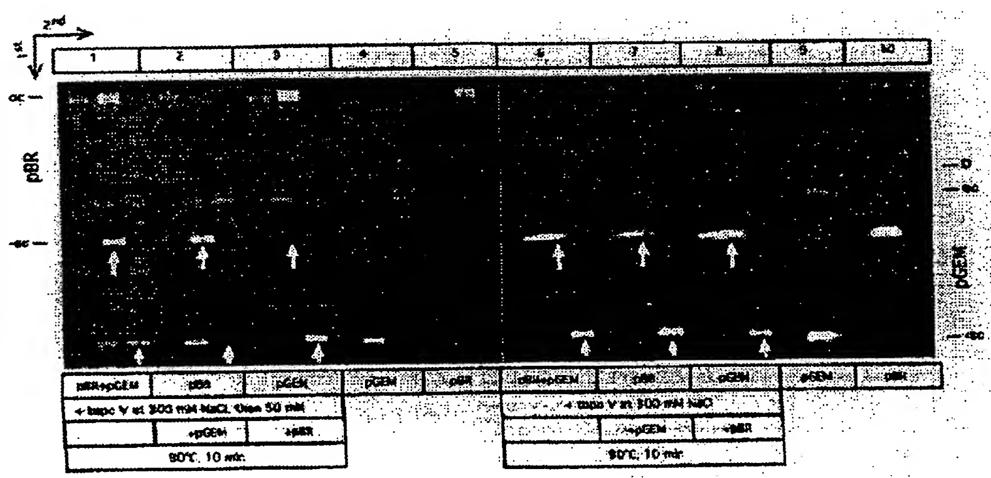


FIG. 14B

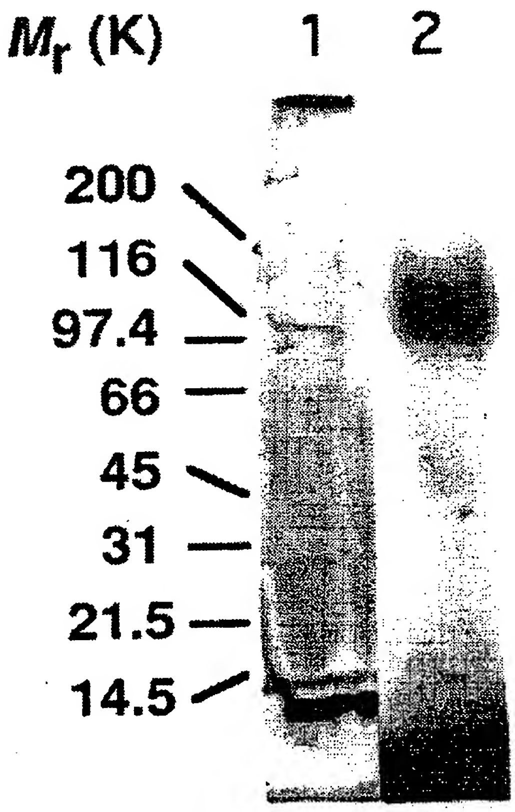


FIG. 16

FIG. 17

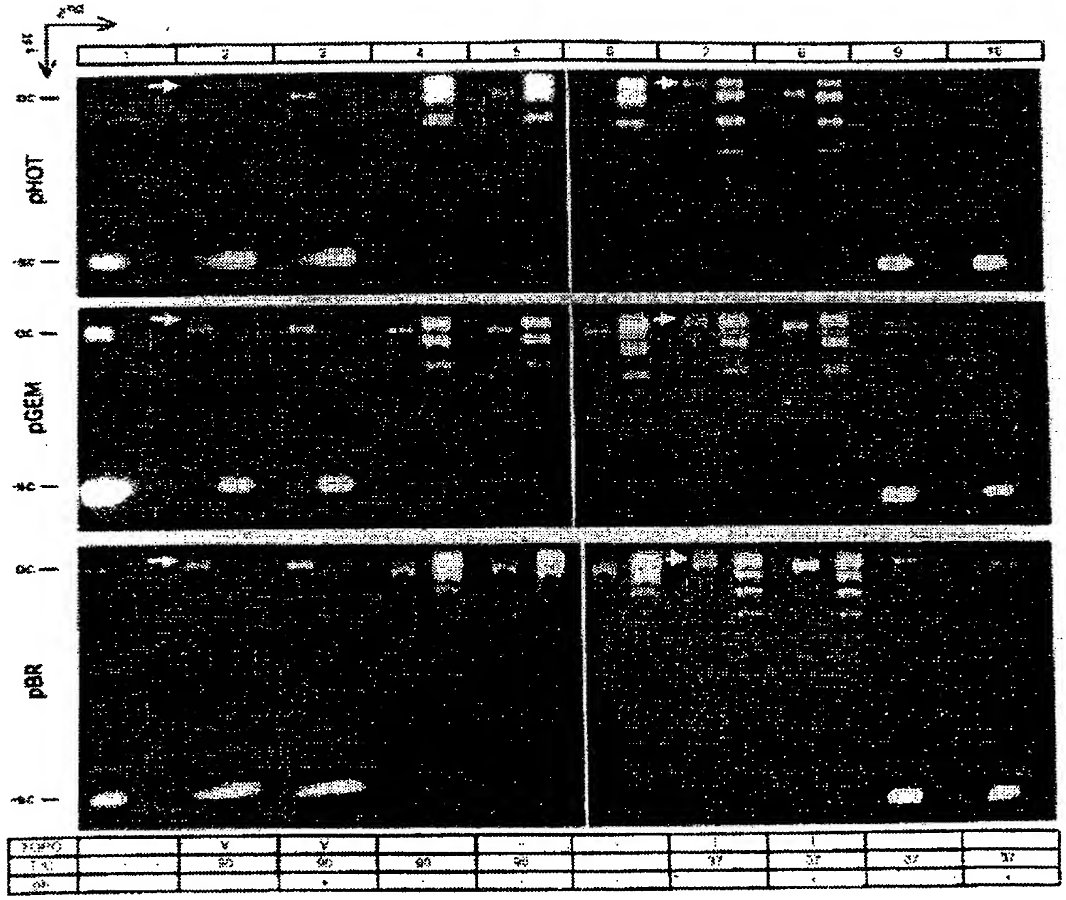


FIG. 19

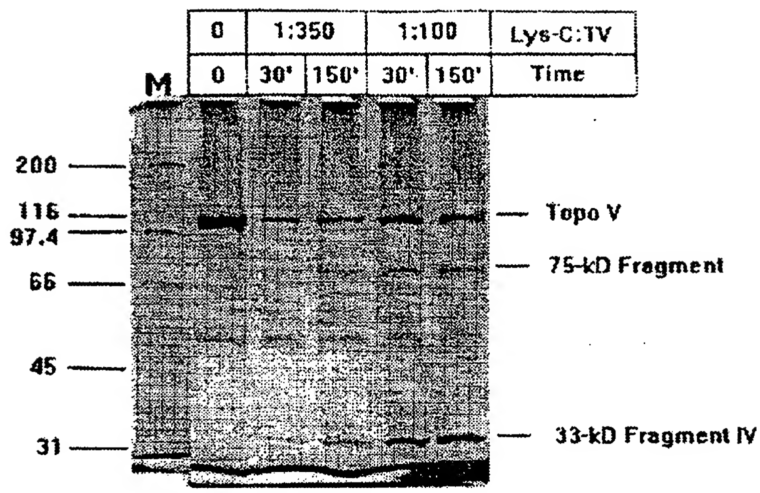

0
1:350
1:100
Lys-C:TV
0
30'
150'
30'
150'
Time
M
200
116
97.4
66
45
31
Topo V
75-kD Fragment
33-kD Fragment IV

FIG. 20

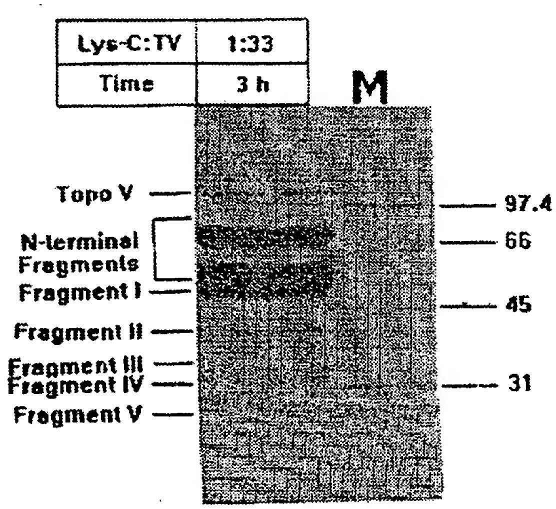

Lys-C:TV
1:33
Time
3 h
M
Topo V
N-terminal Fragments
Fragment I
Fragment II
Fragment III
Fragment IV
Fragment V
97.4
66
45
31

FIG. 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03217

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) : C12P 19/34; C12N 9/90; C07H 19/00
US CL : 435/91, 233; 536/22.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/91, 233; 536/22.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PIR 36, SWISS PROT 25, AGENSEQ 11, EMBL 7, GENBANK 77, REGISTRY, CAS, BIOSIS, EMBASE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	N.R. Cozzarelli et al., "DNA TOPOLOGY AND ITS BIOLOGICAL EFFECTS", published 1990 by COLD SPRING HARBOR LABORATORY PRESS, (COLD SPRING HARBOR, N.Y.), pages 41-57,see Chapter 11, pages 41-57 by J.C. Wang et al.	1-2, 4-6, 17-18
Y	J. BACTERIOLOGY, Vol. 173, No. 12, issued June 1991, C. Bouthier de la Tour et al., "Reverse Gyrase in Thermophilic Eubacteria", pages 3921-3923, see entire document.	1-6, 8-11, 17-18
Y	NUCLEIC ACIDS RESEARCH, Vol. 18, No. 9, issued 11 May 1990, O.I. Kovalsky et al.,"Archaebacterial Reverse Gyrase Cleavage-Site Specificity Is Similar to That of Eubacterial DNA Topoisomerases I", pages 2801-2805, see entire document.	1-6, 8-11, 17-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 MAY 1994	JUN 15 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JON P. WEBER, PH.D. D. Kryza for
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03217

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. BIOLOGICAL CHEMISTRY, Vol. 266, No. 19, issued 05 July 1991, A.I. Slesarev et al., "DNA Topoisomerase III From Extremely Thermophilic Archaebacteria", pages 12321-12328, see entire document.	1-6, 8-11, 17-18
Y	TRENDS IN BIOCHEMICAL SCIENCES, Vol. 10, issued September 1992, D.A. Cowen, "Biotechnology of the Archaea", pages 315-323, see entire document.	1-6, 8-11, 17-18
Y	NATURE, Vol. 342, issued 14 December 1989, R. Huber et al., "A Novel Group of Abyssal Methanogenic Archaebacteria (Methanopyrus) Growing at 110 °C", pages 833-834, see entire document.	1-6, 8-11, 17-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03217

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-6, 8-11, 17-18

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. a. Claims 1-2, 4 and 8-11, drawn to a first product, topoisomerase V from Methanopyrus kandleri per se, classified for example, in Class 435, subclass 233,

b. Claim 3, drawn to a first method of making the first product, topoisomerase V, classified, for example, in Class 435, subclass 233,

c. Claims 5-6 and 17-18, drawn to first methods of using the first product, topoisomerase V, to modify DNA, classified, for example, in Class 435, subclass 91.

II. Claim 7, drawn to a second product, a complex of DNA and topoisomerase V, classified, for example in Class 435, subclass 233 and in Class 536, subclass 22.1+.

III. Claims 12-16, drawn to a third product, antibodies to topoisomerase, and methods of using the antibodies to detect topoisomerase, classified, for example, in Class 424, subclass 85.8, Class 530, subclass 388.26, and Class 435, subclass 7.4+.